The University of Texas Publication

No. 5109 May 1, 1951

BIOCHEMICAL INSTITUTE STUDIES IV

Individual Metabolic Patterns and Human Disease: An Exploratory Study Utilizing Predominantly Paper Chromatographic Methods

From the Biochemical Institute and the Department of Chemistry, The University of Texas and the Clayton Foundation for Research, Austin.

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The benefits of education and of useful knowledge, generally diffused through a community, are essential to the preservation of a free government.

Sam Houston

Cultivated mind is the guardian genius of Democracy, and while guided and controlled by virtue, the noblest attribute of man. It is the only dictator that freemen acknowledge, and the only security which freemen desire.

Mirabeau B. Lamar

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Acknowledgement

The authors wish to express their grateful thanks to the many individuals and organizations whose cooperation and help have made possible the investigations described in this bulletin.

To Drs. Lorene Lane Rogers and Roy C. Thompson and to Mr. Jim S. Berry we acknowledge extensive help in the preparation of a number of the papers in addition to those on which their names appear as co-authors. Without this help the publication of the material would at best have been materially delayed.

Our especial thanks go to Dr. A. T. Hanretta and the Staff of the Austin State Hospital for their kind cooperation in connection with the studies of mental patients; to Dr. C. W. Castner and the Staff of the Austin State School for helping with the difficult problem involving the mentally deficient children; and to members of the Staff of the Austin Public Schools and of St. Mary's Cathedral School for help in connection with the control children. We wish to express our gratitude to Dr. Pearl S. Swanson, Iowa State College, to Dr. W. F. Dunning, Detroit Institute of Cancer Research, and to Dr. W. F. J. Cuthbertson, Glaxo Laboratories, Middlesex, England, who were kind enough to make available to us highly inbred strains of rats. We also express our appreciation to all those who, often with inconvenience to themselves, have served as subjects for the investigations, and to all other members of the Biochemical Institute Staff who have cooperated in numerous ways.

For financial assistance on minor aspects of the extended study we gratefully acknowledge help from the Office of Naval Research in Washington, and from Research Corporation and the Nutrition Foundation, both of New York.

THE AUTHORS

I. Introduction, General Discussion and Tentative Conclusions

by

ROGER J. WILLIAMS

When the writer published in 1946 the volume *The Human Frontier* (1), he had become convinced that in many areas of human interest a careful study of individual people without undue haste in trying to make generalizations would contribute enormously to human understanding and the ability to deal successfully with members of the species known as *homo sapiens*.

Too many studies, he felt, were founded on the assumption that there is such a creature as *the normal man* and that every energy must be directed toward ascertaining the characteristics possessed by this creature. He became convinced that the average man is in a sense a chimera and that further progress will come through an understanding of real individuals and developing the ability to classify them in relation to the various problems which they encounter. Generalizations with respect to human beings are very much to be desired, but unless the generalizations are backed by adequate information, they are premature and superficial and liable to be incapable of substantiation.

The writer envisaged and still envisages the time when real people will be studied from any and every angle and when no promising discipline will be ignored in connection with such studies. There are hopeful moves in this direction, the most important of which is embodied in the Report of the Trustees of the Ford Foundation (1950). This report avoids completely the tendency to compartmentalize all human knowledge; instead it recognizes the existence of human problems and of basic knowledge and insight needed for their solution—knowledge and insight which must often come through the agency of a number of different disciplines. One of the five broad areas which this Foundation will be concerned with supporting is the development of an interdisciplinary science of man. Another has to do with educating individuals to their full capacities, which will entail (interdisciplinary) methods for determining individual potentialities.

Before an all-round, well-directed inquiry into the nature of human beings became possible, we decided in our laboratories that

within our own area of specialization—biochemistry—much could be done in line with this point of view which would of itself be valuable and at the same time would help pave the way for broader studies involving other disciplines.

It was decided that support would most likely be forthcoming and the most would be accomplished if a specific well defined problem were uppermost in our minds. After due consideration the problem decided upon was alcoholism. It appeared highly probable in this case that an exploitation of the *biochemistry of individuals,* in contrast to the *biochemistry of normal man,* could yield valuable and useful information.

However, we realized that before we could gain any significant information about the distinctive biochemistry of those afflicted with alcoholism (or any other group) we would have to gain a background of biochemical information regarding individuals in general.

While this approach was under way we were fortunate in discovering a nutritional attack on the problem of alcoholism which was not anticipated at the outset. This has been pursued with highly favorable results, which have been reported elsewhere (2, 3).

Attempts to find metabolic traits or trait patterns which would be distinctive for alcoholics have been continued; this has necessitated studying individual metabolic patterns in general and in turn has led to the preliminary study of groups other than alcoholics—namely schizophrenic individuals, feeble minded individuals, obese and lean individuals, young children and, to a very limited extent, identical twins.

General Discussion of Results

The exploratory nature of the studies here reported must be emphasized. We became convinced that an exploration of individual metabolic patterns was an urgent need, and were determined that we would carry out such an exploration with whatever means we could command. If exploration in a refined manner were impossible, then we would adopt cruder methods. If highly accurate measurements could not be taken, we would make rougher measurements. If the best results could be obtained only by having all our subjects hospitalized (clearly an impossible undertaking from our standpoint), we would be content with something less than the best. As our work proceeded we could see that by taking short cuts that ordinarily might be frowned upon, we could gather

significant or at least highly suggestive data, whereas if we meticulously and exhaustively developed all the methods underlying each individual item, our exploration would be too limited in extent to be useful or worthwhile.

One of the factors which forced us into the use of "rough and ready" methods with respect to some items was the tremendous volume of work which needed to be done. We have estimated that in connection with this investigation approximately 200,000 paper chromatograms have been run. Allowing only one analytical result for each chromatogram (which is highly conservative) this would mean an average of about 700 analyses each working day for a year, by this method alone. To have carried out anything approaching this number of analyses by long drawn out methods would have been absolutely prohibitive from the standpoint of space, money, and manpower.

Two extremely favorable aspects of the situation have made it possible to get significant results: one is the very timely development of paper chromatography; the other is the fact that differences observable between individuals are often gross and require no hair-splitting refinements to detect. Corresponding values obtained from different individuals often differ not by 10 or 20 per cent but by as much as several hundred per cent.

The meaning of the term metabolic pattern needs to be clarified. Insofar as the total picture of the metabolism of an individual (including the chemical processes in each and every organ and tissue and their effects on each other) is distinctive, it constitutes his metabolic pattern. The complete metabolic pattern of any individual would probably be an exceedingly complicated matter, and of course our studies are only exploratory in this field. We have merely dipped here and there into the vast area and have limited ourselves to items that could be measured with relative ease. The items which we include in our fragmentary metabolic patterns are not necessarily the most striking items nor by any means the most important ones; they are simply items which show distinctiveness and which we are able to measure practically by objective means and without inconveniencing the subjects.

It would be a most interesting phase of the subject if one could measure quantitatively in individuals the basal output of the various hormones coming from the various glands of internal secretion. This information alone would probably be far more revealing than all that we have been able to gather, but of course science has not developed to the point where this can be done. We have

to be content at this time with those items which can be measured by a reasonable expenditure of effort.

Particularly for those outside the field of biochemistry it will be desirable to make clear that metabolic patterns have very little relation to so-called basal metabolism or basal metabolic rates. Basal metabolism values are crude summations of the total energy yielding reactions of all the tissues and organs of an individual, and reveal nothing as to the pattern or details of the metabolic mechanisms. Two individuals might have exactly the same total metabolism, measured in calories, and yet have metabolic patterns differing widely from one another.* Conversely, two individuals might have metabolic patterns very much alike and yet possess total metabolisms very different from one another.

For reasons of convenience we have studied principally the compositions of urine and saliva samples. We would have preferred complete 24 hour specimens of urine to the morning samples which we have most often used, but we found it impractical except in a limited number of cases to obtain these. Again we would have liked to have repeated samples of blood for analysis, but we could not expect our voluntary subjects to undergo repeated inconvenience and annoyance, and hence our studies of blood composition have been limited.

Another series of items which has been studied in some of our subjects is taste thresholds. These have been included only in a limited number of cases where we had complete cooperation of the subjects. How these thresholds are related to internal metabolism is not well known, but in the case of taste sensitivity to sodium chloride, for example, there is a definite physiological connection.

The following charts depict in summary form some of our findings. In Figure I is charted in polar co-ordinates for a hypo-

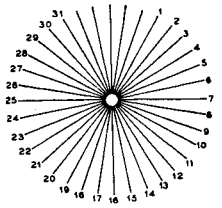
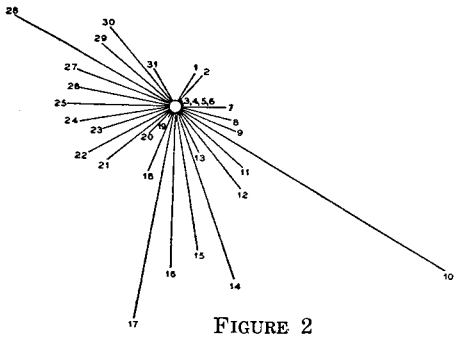


FIGURE 1

*To attempt to get information about the metabolic pattern of an individual by studying his basal metabolism might be compared to attempting to determine the pattern of a wall paper by measuring the light intensity in the center of a papered room.



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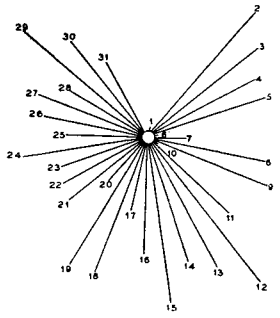

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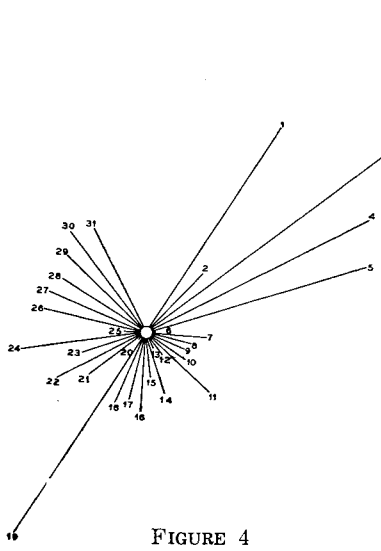

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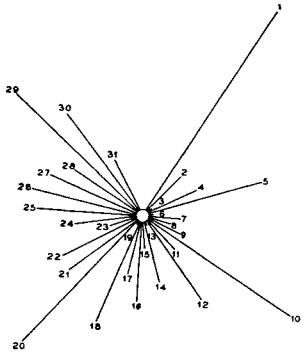

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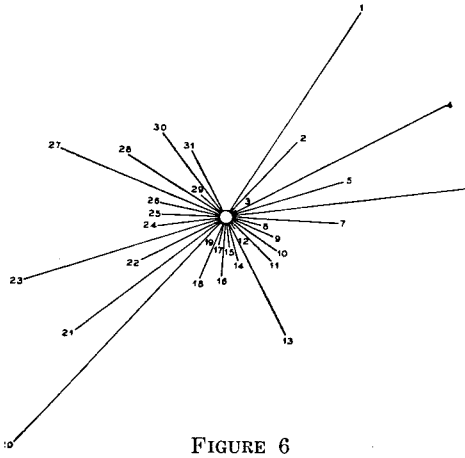

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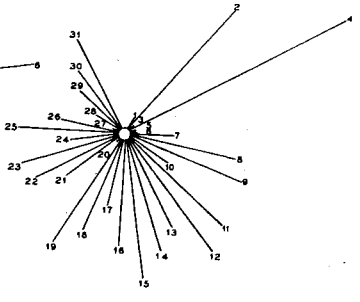
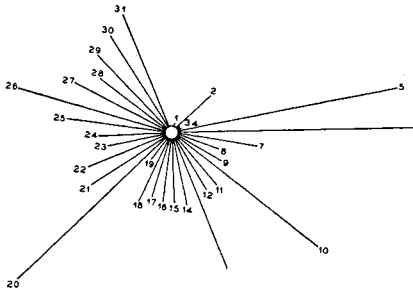


FIGURE 7

(FIGS. 1–13). Taste Sensitivity: 1. Creatinine, 2. Sucrose, 3. KCl, 4. NaCl, 5. HCl. Salivary Constituents: 6. Uric acid, 7. Glucose, 8. Leucine, 9. Valine, 10. Citrulline, 11. Alanine, 12. Lysine, 13. Taurine, 14. Glycine, 15, Serine, 16. Glutamic acid, 17. Aspartic acid. (contd. p. 6)



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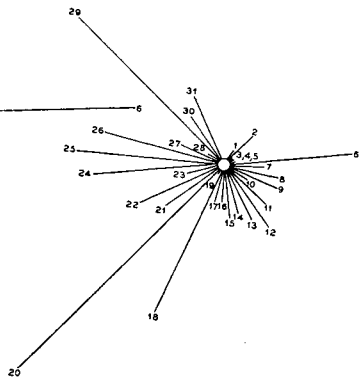

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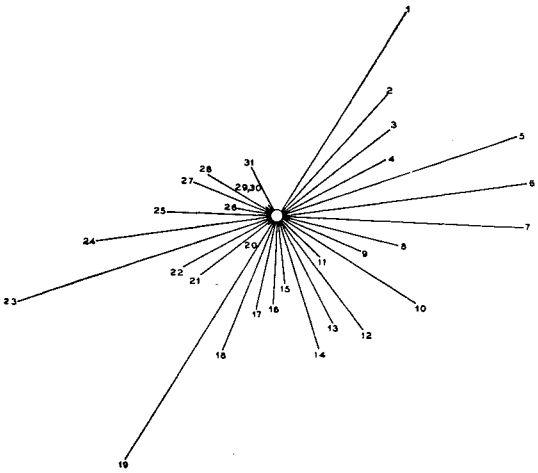

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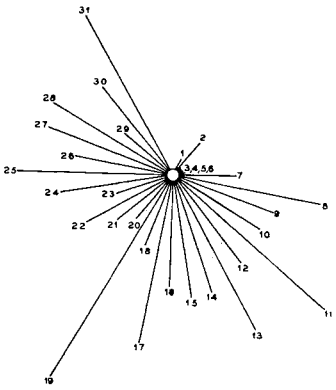

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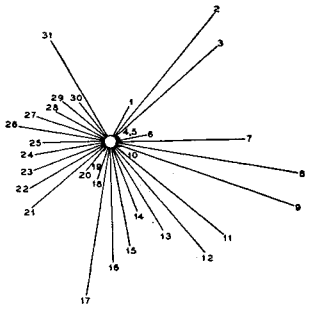

FIGURE 12

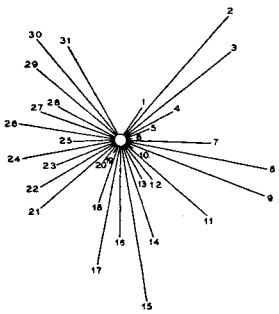

FIGURE 13

(FIGS. 1–13). (contd.) Urinary Constituents: 18. Citrate, 19. Base Rf.28, 20. Acid Rf.32, 21. Gonadotropin, 22. pH, 23. Pigment/creatinine, 24. Chloride/creatinine, 25. Hippuric acid/creatinine, 26. Creatinine, 27. Taurine, 28. Glycine, 29. Serine, 30. Citrulline, 31. Alanine.

thetical individual, various numbered items which we are able to measure. The hypothetical individual charted is *one all of whose measurements are average,* and as will be noted in Figures II-XIII, which represent corresponding charts for twelve actual individuals, the hypothetical individual's chart bears no resemblance to that of any of the actual individuals. It may likewise be noted that there is no such thing as a "normal" pattern.

It appears that each individual that we have studied has whenever tested exhibited a characteristic pattern of measurements which is distinctive for that individual alone. While there are in every case day-to-day variations in saliva and urine compositions and in taste thresholds, certain items, at least, stand out as grossly distinctive, and the patterns as a whole remain nearly constant.

Several questions naturally arise in connection with these and more extended "patterns." How are the patterns influenced by diet? If diet is an important factor, to what extent is the self selection of diets on the part of individuals important? To what extent do special foods or medication contribute to the production of the patterns? To what degree is the genetic background of the individuals a determininative factor in producing the distinctive patterns? To what extent is the characteristic pattern of urine composition merely a reflection of differences in kidney functioning, and to what extent does it indicate more fundamental metabolic differences?

None of the above questions can be answered with certainty and definiteness, though our continued investigation has given indications, and in some cases very strong indications, as to what the answers are.

In the publication of Kirby and Thompson (4) it was reported that the patterns of urinary amino acid excretion with respect to four amino acids, leucine, lysine, threonine, and arginine, were substantially unchanged when each of the five individuals subjected to study was placed for four days on a uniform Army K ration diet. This finding shows that not all the differences, at least, can be ascribed to diet. It does not prove of course that every distinctive item which is discoverable when individuals are on uncontrolled diets, will prove to be distinctive regardless of dietary changes.

A further study was made later (p 173) which sought to answer the same question. The results are not conclusive with respect to all the items measured, but for most of the items it appears that

shifting the individuals from an uncontrolled diet to one in which they all ate the same selection, did not cause material alteration in their excretion patterns. A very substantial amount of research will have to be done before it can be stated with assurance, with respect to all the items, whether or not they are influenced by ordinary changes in the diet. Common medicinal agents such as salicylates or sulfa drugs give rise to characteristic spots on chromatograms, but they do not interfere with the determination of amino acids and other metabolites which constitute the more important items in our patterns. We can be reasonably sure that a considerable number of the items we have measured remain constant for a given individual and that the differences are not due primarily to peculiarities in the diets of the individuals concerned.

Evidence of quite a different sort supports the conclusion that metabolic patterns are distinctive for individuals and are not revolutionized by changes in diet. Bloodhounds for example are able to follow the scent of an individual regardless of what his diet may have been. We are unaware of any observations indicating that dogs are confused in their identification of human beings (or of other dogs) by changes in diet on the part of the individuals to be identified.

It is obvious, however, that certain items which we have measured, such as chloride excretion, must be dependent on the diet. A high percentage of chloride excretion takes place through the kidneys, and it is reported that human consumption of salt may vary from 2-30 grams daily (5). Obviously anyone who consumes a relatively large amount of salt must excrete a relatively large amount in the urine. The fact that excretion patterns are influenced by diet, however, does not by any means prove that the patterns lack significance. Though we do not know what the significance is, it seems most unlikely that one person could spontaneously eat 30 grams of salt a day and another 2 grams per day, without the fact being physiologically significant.

It is conceivable (though not in accord with our findings) that individuals could possess significant individual urinary patterns, based entirely upon their own distinctiveness with respect to food selection and consumption. Notwithstanding the fact that members of the general population (at least within a cultural level) will often accept and partake of identical meals, the resemblance between what two associated individuals eat is probably more apparent than real, because servings are by no means always eaten in their entirety and even when the main items in the menus

are identical, there still may be choices with respect to bread and butter, beverage, condiments, etc. Furthermore, individuals who are closely associated may have become so partly because of their similarity in tastes, and even though they may eat some individual meals that are identical, they often have chances to choose differently—at other meals and between meals. It may be that self selection of food by individuals is more important than is now appreciated, in connection with the whole problem of proper nutrition. Obviously as has been pointed out elsewhere (3), however, self selection cannot be relied upon as an adequate guide to good nutrition.

A number of our studies and findings are related to the question of the importance of hereditary backgrounds in the determination of metabolic patterns as we have measured them. One of the findings is that experimental rats *on identical diets* often show consistent differences in their urinary excretion patterns. These can be ascribed to no cause of which we are aware, other than hereditary differences. Furthermore our extensive studies of individual rats on different dietary regimes with respect to their alcohol consumption, have resulted in a multitude of observations which can only be explained on the basis of the possession of distinctive patterns which depend on the genetic backgrounds of the individual animals.

Turning to human experiments, one of the studies bearing on the point of the roles of diet and heredity is that having to do with young children. The diet of very young children, consisting mainly of milk, may be regarded as relatively uniform. Yet in young babies as in older children and adults we observe the existence of distinctive urinary excretion patterns. In view of other evidence this finding points strongly to the important role which heredity probably plays in the determination of these patterns. In the same study a further evidence of the importance of genetic factors was gained by comparing statistically the excretion patterns of three brothers with each other and with non-siblings. The patterns of the three brothers showed sufficient similarity to suggest strongly inheritance as the basis.

Another type of study which should throw much light on this problem involves the metabolic patterns of so-called "identical" twins. Our investigation in this area has been limited. However, Figures XII and XIII (p. 12) are charts for two "identical" twins, and mere inspection reveals a likeness which is not observed among the others none of whom are twins.

In connection with such twin studies it should be made clear that while the term *homozygous* as applied to twins has a definite meaning, namely that the twins were derived originally from a single egg cell, one cannot conclude that such twins are completely identical in their inheritance. Partial asymmetry reversals are observed, and perhaps related to these is the fact that in some cases homozygous twins are very much alike in appearance and otherwise, while in other cases they differ substantially. Due to mechanisms not well understood and not based on known morphological sequences, homozygous twins differ in the degree to which they are alike. These differences, certainly those involving partial asymmetry reversals, can hardly be attributed to nutritional differences during fetal life.

The homozygous twins which were available for our limited study differed in appearance slightly, particularly with respect to the curliness of their hair, and hence from the standpoint of appearance they were not as much alike as so-called identical twins sometimes are. It should not be a matter of surprise therefore that their metabolic patterns showed some differences. It should be noted that their diets were not controlled.

Our exploratory studies designed to find out whether individuals who have a strong tendency to become alcoholic (actually members of Alcoholics Anonymous, during periods of abstinence) possess metabolic features which are distinctive, resulted in positive findings (6). The evidence gained in this study greatly strengthens the thesis that metabolic patterns of individuals are not only distinctive, but are significant and important in connection with certain human problems.

The question may well be asked in connection with this study: Do the individuals have distinctive patterns which cause them to become excessive drinkers, or have their patterns resulted from excessive drinking? While we cannot categorically answer this question on the basis of available information, it seems to us very unlikely that excessive drinking would cause a change in pattern which would remain changed for many months after the drinking ceased. The whole background of information on metabolic patterns which we possess fits in well with the hypothesis that the tendency toward excessive alcohol consumption has as a forerunner the possession of a characteristic metabolic pattern.

The study of schizophrenic and well individuals, though carried through only to a limited extent, has also yielded positive

results. There are numerous features which appear to be distinctive to a greater or lesser degree, for individuals in this group.

The question here again may be asked: Are the individuals schizophrenic because of the prior possession of characteristic patterns or do the patterns result from schizophrenia? We are unable to answer this question with definiteness since we have not studied the metabolic patterns of individuals both before and after attacks of schizophrenia. Either interpretation of the findings points unquestionably to the physiological basis of schizophrenia.

Our numerous observations lead us to think that the hypothesis of the prior possession of characteristic patterns which predispose toward schizophrenia, is more tenable than the alternative one. Kallman's survey (7) of the subject points very strongly, we believe, to the importance of inheritance in the predisposition toward schizophrenia, and all our findings are in accord with the supposition that metabolic patterns are inherited and remain relatively constant. To what extent they may change with age, is a problem largely for the future which will be discussed briefly in later paragraphs.

Our limited exploration of the metabolic patterns of feeble-minded children as compared with other children not so afflicted has also shown highly significant differences. There can be no question with respect to the importance of inheritance in mental deficiency, and in this case it seems meaningless to consider the question whether the feeble-mindedness may have produced the distinctive patterns since presumably the inherent mental deficiency and the patterns existed from the beginning.

The study of the metabolic patterns of obese and lean individuals has yielded several items, notably urinary phosphate excretion, which appear to be statistically different for the two groups. It is our opinion that further exploration of the metabolic patterns of such individuals is likely to yield still other items which are significantly different.

Tentative Conclusions

Growing out of our preliminary but nevertheless extensive explorations have been various conclusions, some more tentative than others, which will prove valuable as a guide for further investigations. The conclusions outlined below are in substantial accord with each other and with the facts as we know them, and present a rather different picture of the world of human beings

than is customarily entertained. The implications of our explorations are far-reaching, not only into the field of medicine but also into the field of education and into the numerous ramifications of the problem of human behavior.

Distinctive metabolic patterns are inherent in each fertilized egg.—Each fertilized egg cell which is on its way toward being a human being has within it the potentialities to produce in a suitable environment an intricate system of organs and tissues which is distinctive for that particular individual. This distinctiveness is not limited to morphological characters, but the entire metabolic pattern—the ability for carrying out the numerous chemical reactions and the relative efficiency of the various parts of the process—is probably inherent in the original fertilized egg. This conclusion is strongly supported by the work of Beadle and his school in the field of biochemical genetics as well as by our explorations.

Changes in pattern with age are probably inherent.—There is ample reason for thinking that as an organism matures its metabolic pattern changes—perhaps not in all respects—and that the rapidity and to some extent the nature of these successive changes—provided the environment is relatively constant—is determined by the genetic make-up of the original fertilized egg. A striking example of a metabolic change which has its basis in inheritance and yet appears only later in adult life is Huntington's chorea. In this unfortunate disease, the devastating change does not appear until the individual has had time to raise a family and pass on his defect to his progeny, but the potentialities for the change were nonetheless in his make-up continuously throughout life.

The relative abilities of various organs, tissues, and bodily mechanisms to function year after year and to resist wear, is probably also inherent in each individual's make-up. (Environmental factors are also important as we will note later.) When important limiting chemical mechanisms are unusually effective, this may make for long continued operation, whereas relatively ineffective mechanisms (which we know from Beadle's work to be inherited) may be conducive to wear and early breakdown.

There has been a growing appreciation in recent years of the fact that as people grow old, certain of their internal mechanisms tend to wear out first. In other words various parts and functions of the body do not age at the same rate. A large percentage of people who reach the age of 50 have already lost the ability

to produce hydrochloric acid in their gastric juice. An increasing number of people are found who become diabetic in middle life or later. In some cases it is merely that the insulin producing machinery is wearing out. Careful investigations have shown that the tendency toward diabetes is inherited—some inherit the tendency so strongly that they are diabetic while they are still children, others may inherit the tendency so weakly that they do not become diabetic until they reach the age of seventy.

The metabolic patterns of individuals are crucial in connection with their susceptibility to disease.—It is well known—though there has been a tendency in medical circles to ignore the fact—that susceptibility to numerous diseases has a relationship to the genetic background of the individual concerned. In some cases the evidence is clear cut; in others, because of the inherently complicated nature of the genetic machinery, the evidence has not been worked out. Physicians have, often for humanitarian reasons, been loath to dwell upon the inescapable facts of heredity. They continually stress what can be done environmentally. This policy is based upon a praiseworthy attitude, except that it tends to overlook the possibility that understanding the herediary mechanisms may make possible an environmental attack which would not be possible without this understanding.

We believe we have discovered a clear cut example of such a situation in the disease alcoholism. We believe this disease in an individual is related to his metabolic pattern, but that recognition of this fact makes possible a successful treatment by nutritional means (5).

Differences do not denote defectiveness.—There is an unfortunate and quite unwarranted tendency to link together inheritance and defects. People undoubtedly inherit (in an admittedly complex manner) the color of their eyes. Does this mean that everyone whose eyes are not of a standardized color are defective? Not at all. Is the person who has small hands or feet a defective in comparison with one who has large hands and feet? Not at all. There are some types of activities for which large hands or feet may be desirable, and other activities for which they had better be small. Were Jack Spratt and his wife both defective because of their preferences for lean and fat meat respectively? Not at all. The ability to utilize different types of foods may well reside in different individuals, and the family is fortunate which can avoid waste. Is the mathematician who may lack certain musical abilities a defective, likewise the musician who may lack mathematical

abilities? Not at all. There is room in the world for both mathematicians and musicians and the two types of abilities do not need to reside in the same individuals.

Biological variability is a most important fact. Life as we know it could not exist without it. Far more is involved than mere inheritance of "normal" and "defective" make-ups. Each of us possesses an individuality which we should cherish, because it is the real basis for our love of freedom. Because two people are different, we cannot draw the conclusion that one is inferior, the other superior. The biological fact of the differences between sexes does not demonstrate that one sex is superior to the other. The fact that one race is different biologically from another does not mean that one is superior and the other inferior. We cannot say of a musician and mathematician that one is the inferior of the other.

We have previously spoken of the probability that every individual possesses a distinctive metabolic personality (8, 9). Comparing these metabolic personalities is like comparing an infinite variety of wall paper or rug patterns. No two are the same, there is no "normal" pattern, and wide differences can exist without any of the patterns being defective.

Metabolic patterns are probably intimately concerned with our psychological make-ups, our mental abilities and our behavior problems.—One clear example of a metabolic trait associated with mental powers (or lack of them) has been known for a number of years. Certain types of feeble-minded children always excrete in their urine phenyl pyruvic acid, and no person excreting this substance has been found to have ordinary mental powers. The children included in this group constitute a restricted and relatively small proportion of the total number of feeble-minded children. What about the others? Do they have metabolic peculiarities of some other sort? Our investigations answer the question in the affirmative and point the way toward finding out more about these metabolic peculiarities.

In view of the findings of Thurstone that "primary mental abilities" are inherited as individual traits (10) we are led to suppose that some day correlations will be found between metabolic patterns and mental traits. Not only is it to be expected that mental traits will be found related to metabolic patterns, but that all sorts of behavioral problems (strikingly those involving sex, for example) will be found closely tied in with the fundamental and widely inclusive metabolic patterns of individuals—patterns of which up to now we have had only glimpses.

Environmental attacks on specific problems may become possible only when the genetic backgrounds of these problems are recognized.—An extremely encouraging conclusion has been reached in our study of the problem of alcoholism—namely that in spite of its hereditary roots, a nutritional attack may be successful. As indicated elsewhere (3) we are of the opinion that a nutritional attack offers promise not only in alcoholism but also in the treatment of numerous diseases of obscure origin, *even including the devastating mental diseases.* Diseases which have a hereditary origin but are capable of being attacked nutritionally we have designated as genetotrophic in nature (11, 12).

There is nothing in our findings which precludes the use of psychological means for the treatment of disease. Certainly the maintenance of suitable mental hygiene has possibilities in alleviating difficulties arising because of peculiarities in metabolic patterns. Of course, the whole problem of how mental attitudes affect the details of metabolic processes remains largely to be explored. That such changes are brought about by psychological means cannot be denied.

It should be obvious from the foregoing discussion that our study is, without question, a preliminary and exploratory one and that further study of an intensive and extensive nature must follow before many of the questions which have arisen can be answered with definiteness. Our explorations have convinced us that there is a whole new area of insight into human problems, which lies ahead.

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II. Development of Paper Chromatography for Use in the Study of Metabolic Patterns

by

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Before paper chromatography had reached its present state of development an ascending technique was devised in this laboratory (1). This involved the use of the simplest vessels, but nevertheless made possible quantitative work. This procedure has been developed and used continuously in our laboratory since it was devised for the specific purpose of studying metabolic patterns.

Our experience with this simple procedure has been extensive, and while it may not be superior to the use of the descending method utilizing the most modern apparatus, it is an effective technique and one which can be used in practically any laboratory without the purchase of expensive equipment.

Our discussion of procedures is limited to those employed in our laboratory. Reviews dealing with the general field of paper chromatography will be found elsewhere (2, 3, 4, 5). Our procedures will be discussed under the following headings: general techniques, solvents, color developing reagents, compounds studied, complications arising in the analysis of biological fluids and quantitative aspects. The application of these general procedures to the determination of specific substances will be considered in more detail in the papers which follow.

General Techniques

The simplicity of the apparatus and the ease with which large numbers of analyses can be performed are the outstanding features of this method. The solutions to be chromatographed are placed in measured small quantities on filter paper sheets which are stapled in the form of cylinders, care being taken that the edges of the sheet do not touch each other. These paper cylinders are placed upright in Pyrex dishes (10 inch diameter) containing the appropriate solvent for resolution of the chromatogram. Where a number of chromatograms are to be run simultaneously,

cylinders of different diameters may be arranged concentrically, care being taken to prevent their touching at any point. The solvent dish rests on the bottom of a six gallon earthenware crock which is covered with a square of plate glass. By grinding the areas of contact between glass and jar and greasing with desiccator lubricant, an airtight seal can be obtained. However, absolute airtightness appears to be seldom essential, and the greasing of the seal is usually omitted.

Whatman No. 1 filter paper has been employed in all the studies described in this bulletin. This grade of paper has proved to be most satisfactory, particularly in regard to its low cost and to its convenience in permitting the overnight resolution of chromatograms. Whatman No. 4 has been used to some extent, and it possesses the advantage of rapid solvent ascension. However, its greater density not only causes considerably greater diffusion and a generalized increase in Rf values, but also renders the quantitative color comparison method (p. 50) ineffective.

Sheets measuring 18 x 11¼ inches have been used most frequently. Solutions to be chromatographed are placed 1¼ inch from the long edge of the sheet, leaving 10 inches for maximum ascent of the solvent, which is normally allowed to reach the top of the sheet. Under these circumstances, Rf values are very conveniently measured using a transparent plastic ruler graduated in tenths of an inch. It is sometimes possible to use shorter sheets with a consequent saving in the time required for solvent ascension. In the quantitative estimation of urea (p. 88), for example, a solvent ascent of about two inches is sufficient to separate the urea spot from interfering substances.

It is not necessary to remove the chromatogram from the crock at the precise instant that the solvent front reaches the top of the sheet. When the solvent reaches this point, resolution of the chromatogram ceases, and, except for slow diffusion, no further immediate changes occur. Prolonged standing for several hours is, however, to be avoided, since the diffusion may introduce serious errors. In certain acid-alcohol solvents esterification occurs, and secondary solvent fronts appear on prolonged standing.

Solutions to be chromatographed are usually placed on spots at one inch intervals along the sheet. A straight-edge with small "V" shaped notches cut into it at one inch intervals has been found convenient for marking these positions. A pencil run lightly along the edge of this ruler marks the chromatogram, and the light pencil marks do not interfere with the subsequent treatment of

the sheet. Samples are applied to the paper in five microliter amounts, using a capillary pipette* (obtained from Microchemical Specialties Co., Berkeley, California). If larger quantities must be applied to a single spot, they are added in five microliter increments, the spot being allowed to dry thoroughly between each application. The use of a hot plate heated to about 50-60°C. is helpful in hastening the drying. This low temperature has no deleterious effect on the sample itself. By this procedure uniform spots of approximately one centimeter diameter are obtained. Where quantitative deductions of any sort are to be made from the developed chromatogram, it is important that the starting spots be of uniform small diameter.

When large numbers of chromatograms are run daily, the most tedious and time-consuming procedure is the application of the sample to the paper. The efficiency of this operation has been considerably increased by the use of a device for stacking the sheets in such a manner that a technician may remain seated in one position while pipetting samples to a number of sheets. This device is shown in Fig. 1. Each sheet is placed so that the line

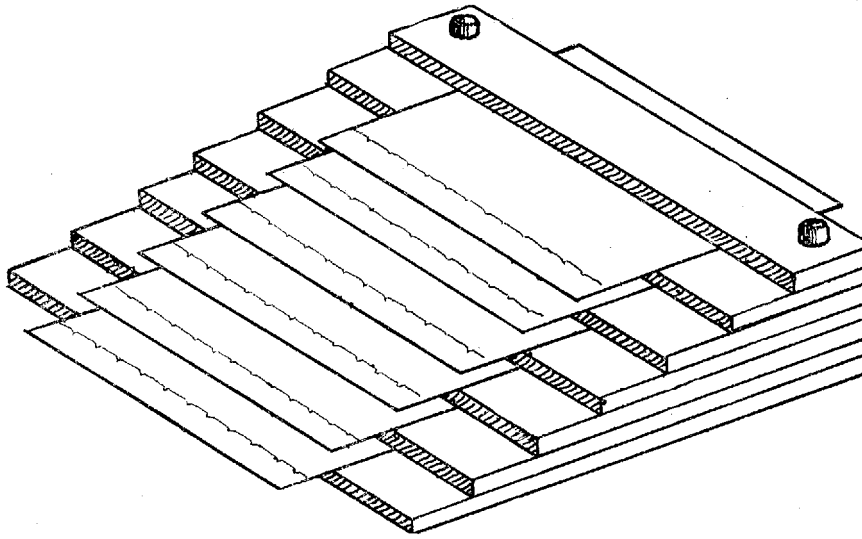

FIG. 1

*A convenient means of cleansing these capillary-bore pipettes is to allow first water and then acetone to be drawn through them by the aspirator. Prolonging the suction 5-10 seconds after the acetone rinse is completed insures drying.

along which the samples are to be placed overlaps the edge of the board under it and does not touch any surface. The sheet immediately above is set back far enough so that it does not interfere with pipetting to the sheet below. As many as eight chromatogram sheets can conveniently be prepared in this manner at one sitting.

Solvents

The various solvent mixtures that have been employed in this laboratory for the resolution of paper chromatograms are described below in the approximate order of their general usefulness. Most of the compounds dealt with have been studied using each of the first five solvent mixtures described. The other solvents listed have also been found generally useful, with the exception of those which are specifically indicated for special purposes. Most of the solvents listed may be used for several determinations, but they should be replaced with a freshly prepared solution after 48 hours. An exception to this rule occurs in solvents in which esterification is likely. In these cases, the solvent mixture should always be freshly prepared immediately before use.

Phenol solvent (*Phen.*) : 100 g. phenol (Mallinckrodt, Analytical Grade) saturated with an aqueous solution containing 6.3% sodium citrate and 3.7% sodium (or potassium) dihydrogen phosphate. About 20 ml. of the aqueous solution are required to saturate the phenol. The salts serve a dual purpose: they inhibit the diffusion of the spots on urine chromatograms, and they prevent the migration of a contaminant in the paper which otherwise obscures the lower half of the chromatogram.

Butanol-acetic acid solvent (*BuAc*) : 80 ml. n-butanol, 20 ml. glacial acetic acid, and 20 ml. water. This solvent must be freshly prepared for each determination.

Butanol-ethanol solvent (BuEt) : 80 ml. n-butanol, 20 ml. 95% ethanol, and 20 ml. water.

Isobutyric acid solvent (*Isobu*) : 80 ml. isobutyric acid and 20 ml. water.

Lutidine solvent (*Lut*) : 65 ml. 2,6-lutidine and 35 ml. water. Lutidine is preferred to collidine because of the greater uniformity of the commercially available product.

Collidine-lutidine solvent (*ColLut*) : 33 ml. of the fraction of mixed collidines boiling between 158 and 165°C., 50 ml. of 2,6-lutidine, and 17 ml. water.

Pyridine-butanol solvent (*PyBu*) : 80 ml. pyridine, 20 ml. n-butanol, and 20 ml. water.

Butanol-pyridine solvent (*BuPy*) : 50 ml. n-butanol, 50 ml. pyridine, and 20 ml. water.

Butanol-ethanol-ammonia solvent (*BuEtAm*) : 80 ml. n-butanol, 10 ml. 95% ethanol and 30 ml. concentrated ammonium hydroxide.

Ethanol solvent (*Et*) : 100 ml. 95% ethanol.

Methanol solvent (*Me*) : 95 ml. abs. methanol and 5 ml. water.

Pyridine solvent (*Py*) : 65 ml. pyridine and 35 ml. water.

Ethanol-acetic acid solvent (*EtAc*) : 95 ml. 95% ethanol and 5 ml. glacial acetic acid. This solvent must be freshly prepared before each determination.

Ethanol-ammonia solvent (*EtAm*) : 95 ml. 95% ethanol and 5 ml. concentrated ammonium hydroxide.

Butanol-ethylene glycol-hydrochloric acid solvent (*BuEGHCl*) : 80 ml. n-butanol, 20 ml. ethylene glycol, and 20 ml. 0.1 *N* hydrochloric acid.

Phenol-acetic acid solvent (*PhAc*) : Same as phenol solvent except that 0.5 ml. of glacial acetic acid is added to the bottom of the earthenware crock to maintain an acid atmosphere. This solvent is useful in separating acidic substances.

Phenol-ammonia solvent (*PhAm*) : Same as phenol solvent except that 0.5 ml. of concentrated ammonium hydroxide is added to the bottom of the earthenware crock to maintain an alkaline atmosphere. After 24 hours a pink discoloration is detectable in the solvent mixture, and the solution must be discarded. This solvent is useful in separating basic substances.

Butanol-ethanol-hydrochloric acid solvent (*BuEtHCl*) : 80 ml. n-butanol, 20 ml. 95% ethanol, and 40 ml. 2 *N* hydrochloric acid. This solvent is especially useful in the determination of cystine and histidine.

Ethanol-hydrochloric acid solvent (*EtHCl*) : 80 ml. 95% ethanol and 20 ml. 0.1 *N* hydrochloric acid. This solvent has been used exclusively in the determination of sodium and potassium.

Isobutyric acid-acetic acid solvent (*IsobuAc*) : 80 ml. isobutyric acid, 20 ml. glacial acetic acid, and 20 ml. water. This solvent has been used for the resolution of certain unidentified urinary constituents.

Color Developing Reagents

Listed below are the reagents which have been used at this laboratory for the development of color on paper chromatograms. A few intensely colored compounds may be detected without the

use of a reagent: e.g., picric acid. Other substances may be detected by their fluorescence under ultraviolet light (UV). Whenever possible the reagents to be applied are prepared in alcoholic solution. This permits rapid drying and minimizes spreading of the spots which may occur after application of the reagent. When using aqueous solutions of reagents, particular care must be taken that the reagent be sprayed *lightly* and *uniformly*. Unless otherwise noted, the reagent is applied to the paper by spraying.

Ninhydrin reagent (*Nin.*) (*6*).—This reagent consists of a 0.2% solution of ninhydrin (triketohydrindene hydrate) in water-saturated n-butanol. The reagent, if kept tightly stoppered in a brown bottle, may be used for as long as one month. After having been sprayed, the sheets are heated for five to seven minutes at 90°C. to promote color development. Most amino acids react to give purple spots, although there are notable exceptions. Phenylalanine, tyrosine, and aspartic acid give blue colors; tryptophane, olive-brown; asparagine, brown; proline, yellow. The reagent is not specific for amino acids; certain amines, ethanolamine, peptides, ephedrine, etc., give colors. As little as 0.2 microgram of certain amino acids may be detected. The presence of high salt concentrations may cause rapid fading of the colors and may even prevent color development entirely. The presence of strong acids in the atmosphere or on the chromatograms (as with the butanol-ethanol-hydrochloric acid solvent) causes reddening of the usual purple color and promotes rapid fading.

Ammoniacal silver nitrate reagent (*AmAg*).—Equal volumes of 0.1 *N* silver nitrate and 5 *N* ammonium hydroxide are mixed immediately before use. Chromatograms may be sprayed with or dipped into the reagent. It is advisable that the sprayer be washed immediately after use several times with 5 *N* ammonium hydroxide followed by washing with water to prevent clogging by deposition of silver and insoluble silver salts. Uric acid develops almost immediately as a black spot against a white background. For further development the sheet should be heated for ten minutes at 100°C., thus darkening the background to light brown. Carbohydrates then appear as dark brown spots. Chloride and phosphate produce bleached areas on the paper. The "weak acid" alkaline area (p. 110) produces a spot slightly darker brown than the background, but at high concentrations (50-100 micrograms of sodium acetate) this may also appear as a bleached area. Most of the amino acids cause a slow bleaching of the background color to white or yellow. The bleaching effect of the amino acids appears

to be different from the immediate bleaching of inorganic ions. The latter seems to be due to simple interference with the contact of the reagent with the paper. As little as 0.2 microgram of uric acid and 0.5 microgram of glucose can be detected using this reagent. The amino acids can be seen only at concentrations of 30-50 micrograms; chloride and phosphate are detectable at 5-10 micrograms. The chromatogram becomes very dark after standing for several days, and the originally darkened areas can no longer be detected. This reagent cannot be used for the color development of chromatograms which have been developed in solvents containing phenol unless the phenol is previously removed by drying the sheet for at least 48 hours at room temperature.

Alkaline ferricyanide-nitroprusside reagent (*FCNP*) (*7*).—Equal volumes of 10% sodium hydroxide, 10% sodium nitroprusside, and 10% potassium ferricyanide solutions are mixed. The mixture is diluted with three volumes of water. After standing for about 20 minutes the initial dark color changes to a pale yellow, and the reagent is ready for use. This reagent is quite unstable at room temperature, but may be kept for two to three weeks in the refrigerator without deterioration. Creatinine, guanidine, glycocyamine and arginine give rise to orange colors against a light yellow background. Creatinine gives a blue color. Certain other nitrogenous compounds also respond to the reagent. The colors are stable over long periods providing precautions are taken to exclude phenol vapors during development and contact with phenolic substances afterward, since these cause marked color changes on the chromatograms.

Ferric chloride reagent ($FeCl_3$).—A 1% aqueous solution of ferric chloride is employed. The reagent may be kept for 7 to 10 days in a refrigerator. Sheets must be sprayed lightly to avoid streaking. Spots appear most clearly after the sheet has dried, except in the case of tartaric acid, which is visible as a yellow spot only on the wet sheet. Phosphate and sulfate produce white areas against a light yellow background. Amounts of phosphate and sulfate greater than 10-20 micrograms can be detected. Urea appears as a pale area slightly lighter than the background and can be detected only in fairly high concentrations (30 micrograms). Basic areas are light brown, presumably due to precipitation of ferric hydroxide. Phenol derivatives produce green to purple spots Aspirin appears in urine chromatograms as an intense purple spot. As little as 0.5 micrograms of salicylic acid or acetyl salicylic acid can be detected.

Phenol-hypochlorite reagent (*PHC*) (*8*).—Chromatograms are first sprayed with 5% phenol in 95% ethanol. After having been dried, the sheets are sprayed with 5.25% sodium hypochlorite (Clorox). Colors develop immediately. The background darkens on standing. This reagent reveals a number of common urinary constituents. Urea, at concentrations above about 5.0 micrograms, appears as a yellow-green spot. Chloride, at concentrations as low as 1.0 microgram, produces a blue spot. Basic cations produce yellow areas. Most amino acids at high concentration (25 micrograms) produce blue or green colors. Arginine, however, yields orange, cystine and cysteine, brown, and tryptophane, pink.

If urea alone is to be measured, preliminary spraying of the resolved chromatograms with 1 *N* sodium hydroxide solution prevents the color development of other urinary constituents. For chromatograms resolved in a phenol solvent the preliminary spraying with phenol may be dispensed with. In this case the amount of phenol remaining on the chromatogram must be controlled by rather careful partial drying for eight minutes (±30 seconds) at 90°C. The sheets must be sprayed immediately after this drying.

α-Naphthol-hypochlorite reagent (*NHC*) (*9*).—Chromatograms are first sprayed with a 0.1% solution of α-naphthol in 1 *N* sodium hydroxide. The α-naphthol reagent may be used for as long as one to two months. After having been dried, the sheets are sprayed with a solution consisting of one volume of "Clorox" diluted with one volume of water or ethanol. Urea appears as a blue-green spot. Arginine appears as a red spot against a white background but fades rapidly. Arginine is detected only at levels above 10 micrograms.

Bromcresol green indicator reagent (*BCG*).—This reagent is a typical acid-base indicator, and as such can be used to detect acidic or basic substances on the chromatogram. A 0.04% solution in 95% ethanol is employed. Before using the solution, the color should be adjusted to blue-green with dilute sodium hydroxide solution. The indicator solution is stable indefinitely. Chromatograms developed in acidic or basic solvents must, of course, be thoroughly dried before spraying. Acids give yellow spots against a blue-green background, while bases show intensification of the blue basic color. Acidic areas on urine chromatograms correspond to chloride, sulfate, phosphate, citrate, lactate, and hippuric acid. Urea appears as a slightly basic spot, while the weak acid alkali (p. 111) gives a strong basic spot. Some metal ions, e.g., lead

and copper, give a bright pink color with this indicator. The colors developed are affected by acidic or basic substances in the atmosphere, but are otherwise quite stable.

Diazotized sulfanilic acid reagent (*DSA*).—4.5 grams of sulfanilic acid are dissolved in 45 ml. of 12 *N* hydrochloric acid with warming, and the solution is diluted to 500 ml. with water. A 100 ml. aliquot of this dilute solution is chilled in an ice bath and 100 ml. of a 4.5% solution of sodium nitrite is added. This mixture is allowed to stand for fifteen minutes in the ice bath. This reagent may be kept in the refrigerator without deterioration for one to three days. Immediately before use an aliquot of this solution is mixed with an equal volume of a 10% solution of sodium carbonate. This reagent is particularly useful in the determination of histidine and a number of unidentified substances present in urine. Pink, red, orange, and purple spots are developed against a light yellow background. The colors are stable, but contact with vapors of phenol, collidine, or lutidine causes darkening of the background. The minimum concentration of histidine detectable is about 0.2 microgram.

p-Dimethylaminobenzaldehyde reagent (*PDAB*).—2.0 grams of p-dimethylaminobenzaldehyde are dissolved in 100 ml. of 1.2 *N* hydrochloric acid. Storage in a refrigerator will keep this reagent three to five days without deterioration. Urea and allantoin give bright yellow spots against a white background. The colors do not fade on drying or standing. The minimum concentration of urea which is detectable is 5 to 10 micrograms.

Bromine reagent (Br_2) (*10*).—0.5 ml. of liquid bromine is added to 50 ml. of glacial acetic acid and 50 ml. of water. This reagent in stable for two or three weeks if kept in the refrigerator. After the chromatograms are sprayed with this mixture they are heated in an oven at 90°C. for three to five minutes. Histidine may be detected by this procedure in amounts of 25 micrograms or more. On exposing the damp sheet to ammonia vapor, the brown histidine spot becomes purple and the sensitivity is increased so that 10 micrograms become visible. The histidine spot does not fade. In addition to histidine, a number of unidentified urinary constituents show up with this reagent as pink, brown and green spots. Some of these spots show a bright yellow fluorescence under ultraviolet light as well. Their ability to fluoresce decreases after several days.

Azide-iodine reagent (*AzI*) (*11*).—50 ml. of a 0.1 *N* iodine solution (aqueous solution prepared using potassium iodide to effect

solution) is added to 50 ml. of 95% ethanol. 1.5 g. sodium azide is dissolved in this solution. If kept tightly stoppered in brown bottles, this reagent is stable indefinitely. This reagent is particularly useful for the detection of sulfur-containing amino acids. Methionine, cystine, and cysteine decolorize the reagent immediately, appearing as white areas against a brown background. After an hour these spots show considerable fluorescence under ultraviolet light. These amino acids may be detected in concentrations of about five micrograms or more. Spots must be marked soon after development, because within a few hours the background fades to give a uniformly colored sheet. Rose and yellow spots due to unidentified constituents then appear on urine chromatograms.

Mercuric nitrate-ammonium sulfide reagent (HgS) (12).—Chromatograms are dipped into a 0.25 *M* solution of mercuric nitrate in 0.5 *N* nitric acid and then heated for ten minutes at 80°C. They are next washed by dipping in 0.5 *N* nitric acid and then in water. After having dried at room temperature the sheets are dipped in a solution of one part ammonium sulfide (reagent strength) and four parts water. Purines and pyrimidines give black spots against a white to gray background. The minimum amount detectable is from 5 to 10 micrograms. The spots are permanent.

Alizarin-ammonia reagent (AlAm).—Chromatograms are sprayed with a 0.1% solution of alizarin in 95% ethanol and then exposed to ammonia vapor. The reagent, tightly stoppered, is stable indefinitely. Calcium and sodium salts of weak acids appear as blue to purple spots against a lavender background. Acid areas appear as yellow immediately; the background fades to yellow on standing. All these substances can be detected in amounts as low as 5 micrograms.

Picric acid reagent (Pic).—Chromatograms are sprayed with 0.5 *N* sulfuric acid and heated for one hour at 100°C. They are then sprayed with a 1.3% solution of picric acid in 95% ethanol which is combined immediately before use with one-fifth its volume of 10% sodium hydroxide. Creatine and creatinine appear as orange spots against a yellow background. Allantoin gives a faint orange spot. A number of other urinary constituents cause a slight fading of the yellow background color. The colors developed for creatinine and creatine are permanent. As little as 0.2 microgram is detectable. If creatinine alone is to be determined, the preliminary hydrolytic treatment with sulfuric acid may be omitted.

Hydrolytic reagent (*Hyd*).—Following the procedure outlined above but omitting the picric acid spray, a pink color is developed with indole and a gray color with tryptamine. In addition, several unknown urinary constituents give pink, blue, and green colors.

2,6-Dichlorophenolindophenol reagent (*DCPI*).—A 0.4% solution of 2,6-dichlorophenolindophenol in ethanol is sprayed on the chromatograms. The reagent is stable indefinitely. This reagent acts as an acid-base indicator as well as an oxidation-reduction indicator. Initially the background is blue with acidic areas appearing as pink spots. The most conspicuous spot in urine is chloride (pink). The weak acid alkali spot (p. 111) appears as a darker blue area. Although amino acids do not appear as acidic or basic substances, they become apparent as completely bleached areas in from one hour to several days, depending on the concentration present. Ascorbic acid causes almost immediate bleaching. Creatinine and creatine appear as bleached areas in about 45 minutes. Lactic and hippuric acids, as well as chloride at fairly high concentrations, cause slow bleaching. Urea may appear as a blue area at high concentrations. The bleached areas remain unchanged indefinitely, although the background changes from blue to pink in forty-eight to seventy-two hours, necessitating the marking of the acid areas before this change occurs. Most of the substances mentioned above are detectable in amounts above about 5 micrograms.

Zinc uranyl acetate reagent (*ZnUAc*) (*13*).—5.0 grams of uranyl acetate are mixed with 3 ml. of warm 30% acetic acid and the mixture diluted to 25 ml. A second solution is prepared, consisting of 15 g. of zinc acetate mixed with 3 ml. of 30% acetic acid and diluted to 25 ml. The two solutions are mixed and warmed gently. Sodium chloride (0.1 g.) is added to the warm solution and the reagent allowed to stand for 24 hours. It is then filtered and is ready for use. This reagent is stable indefinitely. It is used exclusively for the determination of sodium (p. 93).

Lead cobaltinitrite reagent (*LCN*) (*14*).—11.5 g. of lead nitrate and 15 g. of sodium nitrite are dissolved in a small amount of water. When these are completely dissolved, 10 g. of cobalt nitrate are added and the solution diluted to 100 ml. with water. This solution is allowed to stand for at least one hour before filtering to remove the orange-brown precipitate. A 1:2 dilution of the filtrate is prepared immediately before spraying. The reagent is stable for one to two days at room temperature. Contact with

the vapor of the reagent while spraying should be avoided. Potassium and ammonium ions produce brown spots against a light yellow background. The determination of potassium by means of paper chromatography using this reagent is reported elsewhere by Beerstecher (15).

2,6-Dichlorquinonechloroimide reagent (DCC).—The reagent is prepared as a 1% solution of 2,6-dichloroquinonechloroimide in 95% ethanol. When stored in the refrigerator the reagent is stable for two to three weeks. On sheets sprayed with this reagent alkaline areas appear as blue spots, uric acid as a yellow spot, and creatinine as a brown spot. On standing several hours certain unidentified substances in urine appear as brown, pink and red spots on the chromatogram. Contact with phenolic substances must be strictly avoided, and sheets which have been sprayed with DCC should be kept apart from those developed with other reagents because of interaction between this reagent and others, especially sulfanilic acid, the ferricyanide-nitroprusside reagent, and *p*-dimethylaminobenzaldehyde.

Aniline acid phthalate reagent (AHP) (*16*).—To 100 ml. of water-saturated n-butanol are added 1.66 g. phthalic acid and 0.93 g. aniline. The reagent is stable for two to three months at room temperature. On sheets sprayed with this reagent and heated five minutes at 100°C., aldopentoses appear as red spots and hexoses as green to brown spots.

Orcinol reagent (Orc) (*17*).—To prepare the reagent 0.10 g. orcinol in 10 ml. of 95% ethanol is added to 90 ml. of 2 *N* hydrochloric acid containing 0.01% ferric chloride. The reagent should be prepared immediately before use. The sheets are sprayed and heated ten minutes at 85-95°C. Pentoses produce green spots. Heating must be carefully controlled to prevent destruction of the paper by the strong acid.

Naphthoresorcinol reagent (NR) (*17*).—To 90 ml. of 2% trichloroacetic acid is added 0.100 g. naphthoresorcinol dissolved in 10 ml. 95% ethanol. The reagent must be prepared immediately before use. The sheets after having been sprayed are heated ten minutes at 85-90°C. Hexoses produce blue and brown colors.

Naphthylethylenediamine reagent (NED).—Chromatograms are sprayed with a mixture of equal volumes of 0.5% sodium nitrite and 0.5 *N* sulfuric acid. After drying, the sheets are sprayed with a 0.1% solution of N-1-naphthylethylenediamine hydrochloride. This reagent is useful in detecting diazotizable amines. No such compounds were observed in a large number of urine chromatograms.

Compounds Studied

Table I is a listing of compounds studied in this laboratory on paper chromatograms. Included in the table are the Rf values obtained for the pure compounds in the various solvents employed, the color developing reagents employed, the color developed, and, where such information has been obtained, the minimum amount of the compounds which can be detected on paper chromatograms using a particular color developing reagent. The abbreviations employed in the table for solvent mixtures and color developing reagents have been indicated in the preceding sections. More details concerning the characteristics of the colors developed, background color of the sheet, fading, etc., can be obtained by referring

TABLE I

PAPER CHROMATOGRAPHIC BEHAVIOR OF COMPOUNDS IN PURE SOLUTIONS

A. Amino Acids and Amino Acid Derivatives

	Rf Values in Common Solvents					*Other Solvents*		*Color Reagent*		*Min. Amt.*
Compound	*Phen*	*BuAc*	*BuEt*	*IsoBu*	*Lut*	*Rf*	*Solvent*		*Color*	*μg.*
alanine	.55	.38	.16	.40	.18	.21	Et	Nin	purple	0.5
						.46	Me			
						.60	Py			
						.53	EtAc			
						.52	EtAm			
						.39	PyBu			
						.22	BuPy			
						.43	BuEtHCl			
β-alanine	.55	.37	.09	.41	.13	.11	Et	Nin	blue	0.5
						.30	Me			
						.41	Py			
α-aminobutyric acid	.65	.45	.42	.53		.18	Et	Nin	purple	
						.60	Me			
						.64	Py			
						.55	EtAc			
γ-aminobutyric acid	.78	.50	.11	.50	.15	.14	Et	Nin	purple	
						.40	Me			
						.39	Py			
						.75	EtAc			
						.44	EtAm			
						.25	PyBu			
						.12	BuPy			
α-aminocaprylic acid	.74	.85		.21	.55	.03, .59	Et	Nin	purple	
						.08, .75	Me			
						.17, .86	Py			

TABLE I—Continued

A. Amino Acids and Amino Acid Derivatives (Continued)

Compound	*Rf Values in Common Solvents*					*Other Solvents*		*Color Reagent*	*Color*	*Min. Amt. μg.*
	Phen	*BuAc*	*BuEt*	*IsoBu*	*Lut*	*Rf*	*Solvent*			
α-aminoisobutyric acid	.68	.48	.44	.54	.25	.36	Et	Nin	purple	
						.70	Me			
						.71	Py			
arginine	.41	.20	.04	.23	.07	.03	Et	Nin	purple	1.0
						.05	Me	PHC	orange	
						.19	Py	NHC	pink	10.0
								FCNP	red	
asparagine	.29	.19	.06	.27	.09	.03	Et	Nin	brown-purple	1.0
						.09	Me			
						.35	Py			
aspartic acid	.07	.24	.04	.20	.09	.03	Et	Nin	blue	0.5
						.40	Py			
						.28	EtAc			
						.15	EtAm			
						.08	PyBu			
						.04	BuPy			
citrulline	.56	.25	.08	.41	.13	.02	Et	Nin	purple	3.0
						.15	Me			
						.44	Py			
						.23	EtAc			
						.33	EtAm			
						.06	PyBu			
						.07	BuPy			
						.32	BuEtHCl			
cysteine	.19	.07		.09	.07	.10	Et	Nin	brown	
	.57			.23		.12	Me	AzI	decolors	5.0
						.08	Py			
cystine	0.08	.08	.02	.10	.06	.03	Et	Nin	brown	1.0
				18		.05	Me	AzI	decolors	5.0
						.03	Py			
						.17	BuEtHCl			
α, γ-diaminobutyric acid	.25	.12	.05	.15	.06	.03	Et	Nin	purple	2.0
						.03	Me			
dihydroxyphenylalanine	.30	.24	.15	.25	.25			Nin	purple	
								$FeCl_3$	green	
diiodotyrosine	.80	.70	.54	.78	.75			Nin	purple	
								DSA	orange	
								Br_2	yellow*	
glutamic acid	.16	.30	.04	.31	.12	.04	Et	Nin	purple	0.5
						.20	Me			
						.45	Py			
						.32	EtAc			
						.10	EtAm			
						.21	PyBu			
						.08	BuPy			
						.40	BuEtHCl			
glutathione	.25	.05		.10	.08	.03	Et	Nin	purple	
						.15	Me			
						.49	Py			

TABLE I—Continued

A. Amino Acids and Amino Acid Derivatives (Continued)

Compound	*Rf Values in Common Solvents*					*Other Solvents*		*Color Reagent*	*Color*	*Min. Amt. μg.*
	Phen	*BuAc*	*BuEt*	*IsoBu*	*Lut*	*Rf*	*Solvent*			
glycine	0.30	.26	.10	.29	.14	.08	Et	Nin	purple	0.25
						.24	Me	PHC	blue	25.0
						.44	Py			
						.38	EtAc			
						.38	EtAm			
						.23	PyBu			
						.10	BuPy			
						.28	BuEtHCl			
glycylglycine	.39	.22	.06	.27	.49			Nin	purple	
hippuric acid	.75	.93	.81			.80	PhAc	BCG	yellow	
histamine	.52	.22	.18	.03 str.	.33	.34	Et	Nin	brown	
						.20	Me	DSA	rose-brown	
						.71	Py			
						.80	BuEtAm	PHC	yellow	
histidine	.55	.20	.08	.29	.11	.31	Et	Nin	brown-purple	3.0
						.15	Me	DSA	red	0.2
						.47	Py	Br_2	brown-purple	
						.12	EtAc			
						.36	EtAm			
						.23	PyBu			
						.12	BuPy			
						.18	BuEtHCl			
						.08	BuEtAm			
homoserine	.47	.30		.40	.20	.13	Et	Nin	purple	
						.34	Me			
						.60	Py			
hydroxy-proline	.59	.30	.09	.37	.22	.10	Et	Nin	yellow-brown	2.0
						.30	Me			
						.60	Py			
isoleucine	.79	.72	.44	.81	.45	.49	Et	Nin	purple	
						.82	Me			
kynurenin	.43	.76		.62 .66	.26			Nin	purple	
leucine	.79	.73	.42	.82	.43	.50	Et	Nin	purple	2.0
						.82	Me			
						.80	Py			
						.82	EtAc			
						.69	EtAm			
						.68	PyBu			
						.51	BuPy			
lysine	.39	.14	.04	.12	.02	.07	Et	Nin	purple	.25
						.04	Me			
						.13	Py			
						.17	EtAc			
						.31	EtAm			
						.02	PyBu			
						.02	BuPy			
						.18	BuEtHCl			

TABLE I—Continued

A. Amino Acids and Amino Acid Derivatives (Continued)

Compound	*Rf Values in Common Solvents*					*Other Solvents*		*Color Reagent*	*Color*	*Min. Amt. μg.*
	Phen	*BuAc*	*BuEt*	*IsoBu*	*Lut*	*Rf*	*Solvent*			
methionine	.73	.55	.29	.70	.35	.30	Et	Nin	purple	1.0
						.31	Me	AzI	decolors	5.0
						.76	Py			
						.25	EtAc			
methionine sulfone	.53	.28	.13	.39	.24	.08	Et	Nin	purple	
						.26	Me			
						.69	Py			
						.27	EtAc			
						.44	EtAm			
						.42	PyBu			
						.21	BuPy			
						.35	BuEtHCl			
methionine sulfoxide	.72	.25	.11	.49	.14	.07	Et	Nin	purple	
						.55	Me			
						.59	Py			
						.29	EtAc			
						.42	EtAm			
						.27	PyBu			
						.11	BuPy			
						.30	BuEtHCl			
norleucine	.84	.74						Nin	purple	
norvaline	.73	.65	.42	.72	.31	.41	Et	Nin	purple	
						.64	Me			
						.74	Py			
ornithine	.27	.15	.05		.06			Nin	purple	
phenylalanine	.78	.68	.30	.74	.49	.54	Et	Nin	blue	2.5
						.53	Me			
						.75	Py			
proline	.85	.43	.18	.61	.24	.20	Et	Nin	yellow	2.5
						.43	Me			
						.62	Py			
serine	0.24	.27	.10	.28	.16	.07	Et	Nin	purple	0.5
						.20	Me			
						.58	Py			
						.28	EtAc			
						.42	EtAm			
						.28	PyBu			
						.13	BuPy			
						.32	BuEtHCl			
taurine	.29	.19	.12	.17	.27	.10	Et	Nin	purple	
						.35	Me			
						.72	Py			
						.18	EtAc			
						.53	EtAm			
						.49	PyBu			
						.22	BuPy			
threonine	.39	.35	.14	.40	.22	.10	Et	Nin	purple	1.0
						.35	Me			
						.65	Py			

TABLE I—Continued

A. Amino Acids and Amino Acid Derivatives (Continued)

Compound	*Rf Values in Common Solvents*					*Other Solvents*		*Color Reagent*	*Color*	*Min. Amt. μg.*
	Phen	*BuAc*	*BuEt*	*IsoBu*	*Lut*	*Rf*	*Solvent*			
trimethylalanine (neo-leucine)	.81	.66	.50	.78		.78	Py	Nin	purple	
tryptamine	.85	.73	.55	.89	.85			Nin	brown	
								FCNP	white	
								DCC	pink	
								PDAB	purple	
								Hyd	gray	
tryptophan	.66	.50	.29	.71	.50	.16	Et	Nin	brown-purple	3.0
						.62	Me	DSA	red-orange	
						.59	Py	PHC	pink	
tyrosine	.52	.45	.19	.43	.45	.18	Et	Nin	blue	3.0
						.42	Me	DSA	rose	
						.82	Py	$FeCl_3$	brown	
						.15	BuEtAm			
						.38	BuEGHCl			
valine	0.64	.60	.29	.70	.29	.41	Et	Nin	purple	2.0
						.65	Me			
						.74	Py			
						.73	EtAc			
						.62	EtAm			
						.58	PyBu			
						.38	BuPy			
						.76	BuEtHCl			
B. Sugars and Sugar Derivatives										
arabinose	.49	.42	.25	.18	.52			AHP	brown	
								Orc	blue	
								AmAg	brown	
fructose	.53	.39	.24	.17	.47			NR	green	
								Orc	green	
								AmAg	brown	
galactose	.44	.16	.18	.12	.43			AHP	brown	
								NR	blue	
								AmAg	brown	
galacturonic acid	.69	.17	.10	.07	.26			BCG	yellow	
								PHC	brown	
glucosamine	.34	.17	.15	.07	.42			Nin	purple	
								AmAg	brown	
glucose	.35	.16	.20	.12	.53			AHP	brown	
								AmAg	brown	0.5
lactose	.37				.35			AHP	brown	
								AmAg	brown	
mannose	.33	.21	.25	.13	.52			AHP	brown	
								NR	brown	
								AmAg	brown	
xylose	.41	.27	.29	.17	.58			AHP	red	
								Orc	green	
								AmAg	brown	

TABLE I—Continued

C. Vitamins and Vitamin Derivatives

Compound	*Rf Values in Common Solvents*					*Other Solvents*		*Color Reagent*	*Color*	*Min. Amt. μg.*
	Phen	*BuAc*	*BuEt*	*IsoBu*	*Lut*	*Rf*	*Solvent*			
p-aminobenzoic acid	.84	.86	.82	.80	.78	.80	PhAc	$FeCl_3$	brown	1.0
								BCG	yellow	
								PHC	brown	
ascorbic acid	.20	.45	.37		.53	.38	PhAc	BCG	yellow	
								AmAg	black	
								DCPI	pink-bleaches	
N^1-methylnicotinamide		.84						UV	dark	
nicotinamide		.77	.75	.90				FCNP	yellow	
nicotinic acid	.95	.79	.94	.80	.93			DCPI	decolors	
pantonine	.83	.43	.39	.62	.24	.45	Py	Nin	purple	
pantothenic acid		.81				.84	PhAc	BCG	yellow	
								PHC	blue	
pyridoxine	.32	.73	.74	.53		.38	BuEtAm	$FeCl_3$	brown	1.0
								DSA	purple	
riboflavin	.83	.26	.15	.53	.73			UV	yellow*	
thiamine	.25	.36	.30			.55	BuEGHCl	DSA	red-orange	
								FCNP	*	
trigonelline				.94				PHC	brown	
D. Alkaloids										
brucine		.70	.53					BCG	blue	
ephedrine	.95	.86	.69	.80	.85			Nin	purple	
hordenin sulfate	.95	.76	.46	.85	.96			Nin	purple-brown	
								DSA	red	
								PHC	brown	
scopolamine	.98	.68	.66		.99			BCG	blue	
theobromine	.81							PHC	green	
E. Purines and Pyrimidines										
adenine	.94	.70	.70			.76	Py	HgS	black	
						.67	BuEtAm	DSA	pink	
guanine	.60	.35	.23	.52		.78	Py	HgS	black	
						.12	BuEtAm	DSA	orange-purple	
						.20	BuEGHCl			
hypoxanthine	.86 .55							HgS	black	
2-methyl-5-aminomethyl-6-aminopyrimidine	.91	.95	.95	.96	.89			AmAg	black	
uracil	.80		.45					HgS	black	
								PHC	green	

TABLE I—Continued

E. Purines and Pyrimidines (Continued)

Compound	*Rf Values in Common Solvents*					*Other Solvents*		*Color Reagent*	*Color*	*Min. Amt.*
	Phen	*BuAc*	*BuEt*	*IsoBu*	*Lut*	*Rf*	*Solvent*			*μg.*
uric acid	.25	.25	.06		.27	.33	PhAc	AmAg	black	0.2
								DCC	yellow	
								PHC	orange	
xanthine	.09	.01	streak			.08	BuEtAm	HgS	black	
						.13	BuEGHCl	DSA	orange	
F. Aliphatic Carboxylic Acids										
adipic acid	.89	.91	.83					BCG	yellow	
citric acid	.22	.40	.45			.35	PhAc	BCG	yellow	
								$FeCl_3$	white	
fumaric acid		.87				.49	PhAc	BCG	yellow	
α-ketoglutaric acid	.38		.38					BCG	yellow	
lactic acid	.70	.85	.69			.70	PhAc	BCG	yellow	
								$FeCl_3$	white	
malic acid	.34	.65	.29					BCG	yellow	
malonic acid	.57	.72	.36					BCG	yellow	
oxalic acid	.26	.30	.14			.35	PhAc	BCG	yellow	
pyruvic acid		.39						$FeCl_3$	white	
succinic acid	.75	.83	.68					BCG	yellow	
tartaric acid	.21	.25	.15			.35	PhAc	BCG	yellow	
								$FeCl_3$	yellow (when wet)	
								AmAg	black	
G. Miscellaneous Organic Compounds										
acetamide	.30							PHC	green	
allantoin	.45	.27	.23	.34				PHC	green	
								FCNP	orange	
								PDAB	yellow	5.0
5(4)-amino-4(5)imidazole carboxamide	.93		.55		.63			PHC	pink	
								Nin	yellow	
anthranilic acid	.90	.93	.93	.92	.74			UV	blue*	
								FCNP	green	
								PDAB	red	
								DCC	brown	
cadaverine	.55	.17	.09	.05	.62			Nin	purple	
catechol		.95		.74		.97	Py	DSA	purple	
						.01	BuEtAm			
						.98	BuEGHCl			
choline	.97	.45	.27	.23	.97			BCG	blue	
creatine	.91	.36	.10	.36	.27			Pic	orange	2.0
								FCNP	red	
								$FeCl_3$	brown	

TABLE I—Continued

G. Miscellaneous Organic Compounds (Continued)

Compound	*Rf Values in Common Solvents*					*Other Solvents*		*Color Reagent*	*Color*	*Min. Amt. μg.*
	Phen	*BuAc*	*BuEt*	*IsoBu*	*Lut*	*Rf*	*Solvent*			
creatinine	.90	.55	.30		.56			Pic	orange	2.0
								DCPI	decolors (after 45 mins.)	
								DCC	brown	
								FCNP	blue	
								$FeCl_3$	brown	
cysteic acid	.03	.06,.09	.00	.02	.12,.18			Nin	purple	
epinephrine	.76	.47	.28	.32	.78			Br_2	yellow*	
								AmAg	black	
								FCNP	red	
								DSA	pink	
								DCC	brown	
								DCPI	decolors	
ethanolamine	.65	.44	.24	.70 streak	.35	.35 streak	Et	Nin	purple	
						.69	Me			
						.74 streak	Py			
gallic acid	.07	.65	.63	.26	.05			AmAg	black	
								$FeCl_3$	purple	
								FCNP	purple	
								DCC	brown	
glycocyamine		.35	.08		.25			FCNP	pink	
guanidine	.66	.54	.25	.77				FCNP	orange	
								$FeCl_3$	green	
								PHC	green	
hippuric acid	.75	.93	.81			.80	PhAc	BCG	yellow	
hydroquinone		.95		.67		.97	Py	DSA	purple-brown	
						.22	BuEtAm			
						.97	BuEGHCl			
indole	.97	.95	.96	.96				$FeCl_3$	red	
								PDAB	purple	
								DCC	red	
								Hyd	pink	
indole-3-acetic acid	.96	.92	.90	.90	.96			Br_2	red-yellow	
								$FeCl_3$	red	
								PDAB	purple	
								DCC	brown	
isatin		.89	.88	.87				FCNP	blue-green	
leucinol	.31	.02	.02	.32				Nin	purple	
								$FeCl_3$	purple	
neurine hydrate	.92	.84	.82		.90			FCNP	red-purple	
								AmAg	black	
phenobarbital	.85							PHC	green	

TABLE I—Continued

G. Miscellaneous Organic Compounds (Continued)

Compound	*Rf Values in Common Solvents*					*Other Solvents*		*Color Reagent*	*Color*	*Min. Amt.*
	Phen	*BuAc*	*BuEt*	*IsoBu*	*Lut*	*Rf*	*Solvent*			*μg.*
phenyl pyruvic acid		.86	.56		.89			$FeCl_3$	blue	
picric acid	.85	.55	.60	.36	.88			none	yellow	
quinine		.81	.77		.86			UV	blue*	
salicylic acid	.85	.95	.55	.75	.72	.88	Py	BCG	yellow	
						.84	PhAc	$FeCl_3$	purple*	5.0
						.28	BuEtAm	DSA	yellow	
						.98	BuEtHCl	UV	blue*	
sulfadiazine	.92	.73	.69	.53	.84			PHC	brown	
sulfaguandine	.80		.42	.43	.78			PHC	orange	
sulfanilamide	.70	.60	.53	.48	.82			PHC	purple	
sulfathiazole	.88	.72	.68	.58	.84			PHC	yellow-brown	
taurine	.29	.19	.12	.17	.27	.10	Et	Nin	purple	
						.35	Me			
						.72	Py			
						.18	EtAc			
						.53	EtAm			
						.49	PyBu			
						.22	BuPy			
thymol			.73	.97				DSA	red	
urea	.78	.55	.52	.75	.43	.49	BuEtHCl	PHC	green	5.0
								PDAB	yellow	5.0
								$FeCl_3$	white	50.0

*Substance appears fluorescent when viewed under ultraviolet light.

to the discussion of the particular color developing reagent in the preceding section. Where the spots exhibiting fluorescence under ultraviolet light have been utilized in our studies this is noted in Table I by an asterisk. Undoubtedly many of the spots not so indicated in the table also fluoresce. Color developing reagents listed in Table I are only those which have actually been used in this laboratory for the detection of the compound in question. In many cases, other color developing reagents listed in the preceding section might have been used. Where more than one color reagent is listed for a given compound, the one listed first is the one which has been found most useful in our experience.

It should be pointed out in connection with Table I that Rf values under conditions ordinarily employed in the production of paper chromatograms, may vary by a few percent. The variation

may be much greater when the same compound is measured in the presence of varying high concentrations of other substances, an effect which will be discussed in detail later. For this reason the absolute Rf values listed in Table I are of less significance than the relative values of different substances with respect to one another, which remain the same in a given solvent. In a few cases two Rf values are recorded for a compound resolved in a given solvent. As will be pointed out later, this may be a real effect in the case of certain compounds capable of existing in different ionic states. In the case of certain of the less common compounds listed in Table I the effect may be due to the presence of impurities in the sample chromatographed.

Table II lists information similar to that found in Table I except that the Rf values reported have been measured on chromatograms prepared from urine samples rather than from solutions of the pure compound. Section A of Table II deals with inorganic ions present in urine. As will be discussed in detail later (p. 49), the paper chromatographic behavior of inorganic ions cannot be defined except in relation to other substances present in the sample chromatographed. Section B lists a number of as yet unidentified substances which appear quite consistently on urine chromatograms.

Complications Arising in the Analysis of Biological Material

While the interpretation of paper chromatograms is a fairly simple process when dealing with pure solutions or simple mixtures, the complexities of most biological fluids serve to introduce many complications. Difficulties arise from the presence, in relatively high concentrations, of substances other than those to which attention is being directed. Thus, the larger amounts of urea and inorganic salts in urine complicate the paper chromatographic study of this material, while in blood the high level of sodium chloride and the large amounts of protein constitute major complicating factors. Saliva is remarkably free of interfering substances, and its paper chromatographic study is relatively simple and straight-forward, even though rather large volumes (up to 0.25 ml.) must be chromatographed to detect many of the constituents.

TABLE II

A. Inorganic Ions in Urine

Ion	*Rf Values in Common Solvents*					*Other Solvents*		*Color Reagent*	*Color*
	Phen	*BuAc*	*BuEt*	*IsoBu*	*Lut*	*Rf*	*Solvent*		
ammonium						.72	EtHCl	LCN	brown
calcium	.05	.10	.19					AlAm	purple
chloride	.25	.25	.20		.30	.17	PhAc	PHC BCG AmAg	blue yellow gray
phosphate	.05	.17	.10					$FeCl_3$	white
potassium	.19		.15			.50	EtHCl	LCN BCG	brown blue
sodium	.19	.30	.15			.30 .68	PhAc EtHCl	ZnUAc BCG	blue* blue
sulfate	.20	.02	.04			.33	PhAc	$FeCl_3$ BCG	white yellow

B. Unidentified Urinary Constituents

1	.44	.42 .60	.55	.17	.80	.44 .89 .82 .37	IsobuAc BuEtHCl Py BuEtAm	Br_2 $FeCl_3$ DCC PDAB DCPI DSA Hyd NED	green; yellow* blue orange-brown red decolors yellow (fades with Na_2CO_3) green blue
2		.26						DSA	pink
3		.65		.21		.91	BuEGHCl	DSA	pink
4		.85		.54		.95 .23 .95	BuEtHC BuEtAm Py	DSA	orange
5		.90		.71		.98 .24 .95 .65	BuEtHCl BuEtAm BuEGHCl Py	DSA $FeCl_3$ PDAB NED AzI	red brown brown pink decolors
6		.21				.30	BuEtHCl	DSA	white*
7		.06						DCC	pink
8		.95						PDAB	purple
9		.80						PDAB	purple (rat urine)
10 (No. 16 on map of amino acids, (p.—)		.68			.30	.60	PhAm	Nin	purple

*Substance appears fluorescent when viewed under ultraviolet light.

Interfering substances manifest themselves in a variety of ways: simple masking of spots, distortion of Rf values, distortion of spot size, multiple Rf values, and streaking. In addition the behavior of inorganic ions may lead to much confusion. These complications will be discussed in the following paragraphs, with especial reference to their effect on the paper chromatography of urine.

Simple interference (masking of spots).—In a given solvent, the Rf values of two or more interfering substances having similar color reactions may be the same. In such an event these substances will be indistinguishable and a different solvent must be used in which the Rf values of the compounds in question are not identical. When separating complex mixtures of similar substances, such as amino acids in urine, it may often be impossible to find a solvent mixture which will not result in interference between certain components of the mixture. In such a case the two-dimensional chromatogram may often be employed to advantage. The application of such a procedure to urine and saliva samples is described elsewhere in this bulletin (p. 72).

When overlapping spots react differently to the color developing reagent employed, confusing color distortion may occur, or the development of color with certain compounds may be prevented entirely. For example, in urine chromatograms prepared using the butanol-acetic acid solvent, the presence of high salt concentrations may completely inhibit the development of the cystine spot with the azide-iodine reagent. When recognized, interference of this type may be overcome by the use of a different solvent mixture or a different color developing reagent.

Distortion of Rf Values.—The presence of other substances in relatively high concentrations may have a very appreciable effect on the Rf value of a compound. This is illustrated in Table II where the Rf values of various urinary constituents in pure solution and in urine are compared. Urea, creatinine, the acidic and basic ions, as well as uric acid, may cause a generalized lowering of the Rf values from the values of pure compounds in the same solvent. In the case of urea, if the Rf value of a pure compound is either .1 or .2 Rf unit higher or lower than that of urea, the Rf value of the substance in the presence of urea may be considerably lower than for the pure substance. Where the Rf value of a compound very nearly coincides with that of an interfering substance present in high concentration, the spot may actually be split and appear partly above and partly below the position occupied by the interfer-

ing substance. The judicious selection of a different solvent mixture can frequently resolve these difficulties.

TABLE III

Comparison of Rf Values of Substances in Urine and in Pure Solution

Compound	*Solvent*	*Rf Values in Pure Solution*	*Rf Values in Urine*
Alanine	Phen	.57	.53
Arginine	Phen	.41	.39
Asparagine	Phen	.34	.30
Aspartic acid	Phen	.07	.05
Chloride	Phen	*	.25
Creatine	BuEt	.21	.10
Creatinine	BuEt	.49	.37
Cystine	Phen	.12	.01
Glutamic Acid	Phen	.21	.23
Glycine	Phen	.33	.28
Histamine	BuAc	.22	.10
Histidine	Phen	.53	.47
	BuAc	.20	.05 and .15
Leucine	Phen	.84	.81
Lysine	Phen	.36	.30
Methionine	Phen	.73	.70
Norleucine	Phen	.84	.84
Norvaline	Phen	73	.73
Ornithine	Phen	.27	.22
Phenylalanine	Phen	.78	.74
Phosphate	Phen	*	.02
Proline	Phen	.85	.82
Serine	Phen	.27	.22
Sodium-potassium	Phen	*	.19
Sulfate	Phen	*	.20
Tartaric acid	BuAc	.45	.24
Threonine	Phen	.43	.39
Urea	Phen	.78	.78
Uric Acid	Phen	.25	.20
Valine	Phen	.77	.73

*See Table IV.

The complete elimination of difficulties of this sort in the case of urine can be accomplished by removal of urea by treatment with urease, and by desalting through use of ion-exchange adsorbents. This is a somewhat laborious and time-consuming procedure and renders the urine particularly susceptible to bacterial decomposition. An additional means of minimizing many of the Rf distortions which are mentioned above is through the use of the phenol solvent. The variations from the Rf values of pure substances, while still present, are much smaller than for other solvents (see Table III).

The most practical procedure, which has been employed routinely in this laboratory for quantitative work involves adding measured small amounts of urine to the standard spots applied to the

chromatograms. Thus a fairly uniform distortion of all Rf values is obtained. A more complete explanation of this technique will be found in the section of this paper dealing with quantitative measurements.

Spot size distortion.—As will be discussed later in detail, the size of a developed spot on a chromatogram is, under properly controlled conditions, indicative of the amount of substance present. This relationship between concentration and spot size may be considerably altered by the presence of interfering substances in the sample chromatographed. In general, the effect is to produce an increase in spot size, although in some cases partial interference in color development, or "crowding" by other substances, may result in a marked diminution in the size of the spot when compared to the pure substance at the same concentration.

In urine the chief offenders are the inorganic ions, chloride, phosphate, sodium and potassium. The effect is probably due to the hygroscopic nature of these substances on paper, which results in localized "waterlogging." This effect may be directly observed during the drying of the chromatograms, the areas of high salt concentration being the last to dry. With many solvents these salts are not very well localized on the chromatogram but form diffuse streaks, with the result that other constituents of urine with Rf values within the rather wide range of these streaks will form diffuse spots or streaks.

This effect is largely eliminated in the case of the phenol solvent by the presence of ammonia or acetic acid in the developing chamber, which serves to localize the inorganic salt spots. Where the phenol solvent interferes with color development a solvent mixture may be employed in which the Rf values of the compounds of principal interest fall well outside the range of the salt streaks.

Multiple Rf Values.—It is ordinarily assumed that the appearance of two spots on a chromatogram indicates the presence of two distinct compounds. Such is not always the case. Two discrete spots may be obtained from a single substance, or, more frequently, diffuse streaks with what appear to be areas of localized concentration. Aronoff has reported (18) that by varying the pH of aqueous solutions of lysine, a plurality of spots can be produced using a saturated phenol-water system. This effect he ascribes to an association between basic lysine ions and phenol. In this laboratory we have noted a somewhat similar effect in the case of urinary histidine using the butanol-acetic acid solvent.

Two separate and distinct spots appear corresponding to histidine in urine, while aqueous histidine solutions result in only a single spot. Addition of histidine to urine previously showing no histidine likewise results in two spots. Whatever the precise explanation for such phenomena, it would seem to involve the possibility for existence of multiple ionic species.

The appearance of two spots as a result of partial interference by a second substance has already been mentioned. Using the butanol-acetic acid solvent, creatinine usually appears coincident with, or slightly below, urea. When fairly large concentrations of both are present (200 micrograms of urea and 25 micrograms of creatinine) creatinine appears as two spots, above and below urea. If the concentration of creatinine is further increased, it will again appear as a single spot above urea. A somewhat similar effect was encountered in separations of malic acid and potassium ion on chromatograms (five inches in length) using the butanol-acetic acid solvent. Color development with bromcresol green indicator reagent disclosed two acid areas, the lower of which was enclosed in a basic ring. Apparently in this instance the positions of malic acid and potassium partially overlap, and the basic potassium spot entraps part of the malic acid. This interference was prevented by employing smaller samples or by the use of a longer sheet for the chromatogram.

If multiple spotting is due to the existence of multiple ionic species, the use of a solvent which incorporates a strong acid or base serves to concentrate the material in a single distinct spot. The use of hydrochloric acid in the butanol-ethanol-hydrochloric acid solvent in the analysis of histidine is an excellent example of this technique. Where the multiplicity of spots is due to interference by a second substance, the judicious selection of a different solvent mixture should remedy the situation.

Streaking.—Diffuse streaks rather than discrete spots are not infrequently encountered on paper chromatograms. This may be due to any of several causes. As was previously noted, localized "waterlogging" due to the presence of a relatively high concentration of inorganic salts may be one cause. The possession of multiple ionic species as in the case of lysine and arginine may also be responsible. Substances which ordinarily migrate as discrete spots may form streaks when present in higher concentrations. Streaking is most frequently observed when using solvents which contain a relatively high percentage of water. Except where it is due to high concentrations of the migrating substances, streaking may be prevented by the use of a different solvent.

Behavior of inorganic ions.—No thorough study has been made in this laboratory of the apparent erratic behavior of many inorganic ions on paper chromatograms. An investigation of the behavior of these ions is certainly needed and would probably do much to establish a sound theoretical basis for understanding paper chromatography. Such an understanding can scarcely be said to exist at the present time. Our interest has been limited largely to the behavior of these ions and their effects on other substances on urine chromatograms.

Unlike most of the organic compounds studied, a particular inorganic ion cannot be expected to migrate consistently to the same position when developed with a given solvent. The position of the anion, to some extent at least, is determined by, as well as determines, the position of the cation. The situation is well illustrated by the data of Table IV. The relative positions of the acidic and basic spots were determined using bromcresol green indicator reagent, and confirmed by specific reagents for the cation or anion involved: silver nitrate for chloride, ferric chloride for phosphate and sulfate, and zinc uranyl acetate for sodium. The effect of an acid solvent (butanol-acetic acid) in localizing anions may be noted as well as in the production in several instances of multiple sodium spots. No completely satisfactory explanation for the observed separation of anion and cation of a strong electrolyte has as yet been proposed. Westall has postulated (19) the formation of a sodium phenolate ion to account for the separation of sodium and chloride ions when run in a phenol solvent. Since the same phenomenon can be observed in solvents in which there is little probability of combination between the salt and solvent, this explanation would not appear to be of general utility. It seems highly probable that something more than simple partition between solvents is involved and that adsorption forces may play a determining role.

For our own purposes the position assumed by these ions on urine chromatograms is of prime importance, and the Rf values for a number of inorganic ions in urine and the color developing reagents employed for the detection have been listed in Table II.

Additional Quantitative Aspects

A number of methods have been proposed for the quantitative estimation of materials separated on paper chromatograms. Some of these involve rather elaborate techniques of elution and colorimetric measurement (20), photo-densitometer measurements (21)

and even electron diffraction (20). Only the simplest procedures have been used at this laboratory—visual comparison of color intensity and measurement of spot area. Compounds which are detectable in very small amounts (0.2 to 2.0 micrograms*) with sensitive reagents, generally show a relation between concentration and color or color intensity, while the spot area remains virtually unchanged over that range. Such compounds may be estimated quantitatively by visual comparison of color intensity in contrast to other compounds which become apparent only in concentrations above three to five micrograms. In the range from five to thirty, or even up to fifty micrograms, the concentration can often be related as a linear function of the logarithm of the spot area. While subject to numerous limitations, these methods are so widely applicable and straight-forward as to be particularly suited to extensive group surveys.

TABLE IV

Rf Values of Inorganic Ions Derived from Various Compounds in Aqueous Solution

Ion Detected	*Compound Chromatographed*	*Rf Values in Following Solvents*		
		Phen	*BuAc*	*BuEt*
chloride	HCl	.19	.20	.15-.50 str.
chloride	NH_4Cl	.27	.17	.16
chloride	NaCl		.18	.13
sulfate	H_2SO_4	.19-.38 str.	.37	.02 -.45 str.
sulfate	$(NH_4)_2SO_4$	.08-.45 str.	.05	.03
sulfate	Na_2SO_4	.05-.39 str.	.03	.11
phosphate	H_3PO_4	.10	.47	.26-.44 str.
phosphate	NaH_2PO_4	.10	.20	.09
phosphate	Na_2HPO_4	.05	.23	.05
sodium	NaOH	.55	.32	.07
sodium	NaCl		.26	.11
sodium	Na_2SO_4		.03 and .23	.11
sodium	Na_2HPO_4	.03 and .45	.20	.03 and .11
sodium	NaH_2PO_4	.03	.13 and .23	.03
sodium	$NaC_2H_3O_2$	.43	.29	.14

Color comparison method.—For a compound to be quantitatively estimated by this procedure, a number of criteria must be met. First, a good color contrast between the spot and background is necessary. Second, there must be a clearly visible gradation in color or color intensity as a function of concentration. Third, the concentration range over which the color gradation is apparent should be at least three- to five-fold for practical utility. Fourth, distortion of spot color and/or spot size by interfering substances must be eliminated or reproduced in the standard. By careful selection of

*An exception to the range given here is the case of urea, in which the color comparison range is from 8 to 32 micrograms.

solvent and color developing reagent, these criteria can be fulfilled for a large number of compounds of biochemical interest.

For the comparison of color intensity to be valid, standard and unknown samples must be treated under as nearly identical conditions as possible. This can best be achieved by running standard and sample adjacently on the same chromatogram. The principal difficulty encountered lies in the frequent presence of interfering substances in the sample (not duplicated in the standard) which may result in distortion of spot color. When such interference cannot be eliminated by the choice of solvent and color developing reagent (as is most often the case when analyzing so complex a mixture as urine), the practice has been followed of adding to the standard a known amount of the sample to be determined. The standard is then subject to the same distorting influences as the sample. This procedure does not wholly eliminate the difficulty, since the concentration of interfering substances in the compared spots will necessarily be different. However, this general method has in many instances proved capable of satisfactory results.

A rather standardized form of paper chromatogram has been employed for quantitative estimation by the color comparison methods, and the exact procedure can perhaps best be described by considering a specific example. Table V illustrates the form of a chromatogram for the determination of urea in a urine sample.

TABLE V

Form of Chromatogram for the Determination of Urea in Urine by the Spot Comparison Method

Spot Position	*Urine Dilution Employed (5 μl. added)*	*Urea Added (μg)*	*Concentration of Urea in Sample Spot Corresponding to Standard Spot (mg./ml.)*
1 (sample)	1:2	0.0	
2 (standard)	1:4	10.0	8
3 (sample)	1:2	0.0	
4 (standard)	1:4	12.5	10
5 (sample)	1:2	0.0	
6 (standard)	1:4	15.0	12
7 (sample)	1:2	0.0	
8 (standard)	1:4	20.0	16
9 (standard)	1:8	12.5	20
10 (sample)	1:4	0.0	
11 (standard)	1:8	15.0	24
12 (sample)	1:4	0.0	
13 (standard)	1:8	17.5	28
14 (sample)	1:4	0.0	
15 (standard)	1:8	20.0	32
16 (sample)	1:4	0.0	

In every case sample spots are placed adjacent to standard spots containing half as much urine as the sample spot. Under these circumstances if a sample spot matches a standard spot, the amount of urea in the urine added to the standard spot will equal the amount of pure urea added to the standard spot. To consider the general case, if x equals the amount of urea present in the urine added to the standard spot, and y equals the amount of urea added to the same standard spot, then the total amount of urea present in this standard spot will be $x+y$. The amount of urea in the adjacent sample spot containing twice as much urine must equal $2x$. Now, if the two spots are of equal color intensity, they presumably contain the same amount of urea, or $2x$ equals x plus y, from which it is obvious that x must equal y. To consider a specific example, suppose that upon development of the chromatogram represented in Table V, spots 5 and 7 were found to match spot 6. The amount of urea present in the urine added to spot 6 must be 15 micrograms. Since 5 microliters of a 1:4 dilution of urine had been added to spot 6, the concentration of urea in the diluted urine was 15 micrograms/5 microliters; the concentration of urea in the undiluted urine was 60 micrograms/5 microliters, or 12 milligrams/milliliter.

The form of chromatogram illustrated in Table V provides not only that all comparisons will be between adjacent spots, but also that in most cases duplicate sample spots will be available for comparison with each standard spot. Where sample spots do not exactly match any standard spot, interpolation is, of course, possible. The exact form of the chromatogram may be altered to fit circumstances. The only essential feature is the alternate arrangement of sample and standard spots with the standard containing a measured fraction of the urine present in the sample spot.

The color comparison method of quantitative estimation can be applied to two-dimensional chromatograms. Because separate sheets must be employed for each dilution of standard and sample, the method is much more time-consuming than for the case of one-dimensional chromatograms. The probability of error is also greater, since comparisons must be made between spots on different sheets, which, despite all precautions, will have been subjected to slightly differing conditions during resolution and color development.

The quantitative method using comparison of color intensity has a number of advantages which make it the preferred method in routine analysis, where a choice between the two methods outlined here is possible. Since the effective range involves very small

quantities, dilute solutions of the substance to be analyzed can be measured. (As little as 10 micrograms per milliliter can be conveniently determined.) In addition to its sensitivity, the method has other advantageous features: the volumes of solution necessary for application to the chromatogram are generally small, and the arrangement of standard and unknown substances side by side facilitates matching. A major disadvantage of the method is the relatively high absolute error imposed by the inability to distinguish between increments of less than about 0.2 microgram in a range of 0.2 to 2.0 micrograms. The rather narrow concentration range over which the method is usually effective, often necessitates use of several dilutions of the solution to be assayed. This is not only time consuming, but contributes an additional source of error.

Details concerning the application of this method to the quantitative estimation of individual compounds will be found in later papers in the bulletin.

Spot area method.—The measurement of the area of a spot on a chromatogram often serves as a satisfactory method of quantitative estimation. Most commonly, the logarithm of the area of the spot is a linear function of the concentration, although this is not always the case. Such a relationship has been noted by others (22, 23). Brimley (23) has derived the relationship mathematically, assuming it to be analogous to the heat-flow equation.

The criteria necessary for the applications of this method are somewhat more flexible than those for the color comparison method. Color contrast, in this case is not so critical, and variations in color intensity are of no importance. The single important requirement is that the spot area increase with concentration. Where the substance has migrated above an Rf of .10 on a ten-inch chromatogram, the logarithm of area is generally, but not always, a linear function of concentration; where migration does not occur or is less than an Rf of .10, the area is frequently a direct linear function of concentration. Interfering substances causing spot area distortion must, of course, be absent or their effect reproduced in the standard. In this laboratory the method has, in general, been limited to those cases where interference does not occur or where it may be eliminated by such simple means as the choice of a different solvent.

Standards and unknowns must receive, as nearly as possible, identical treatment. It has been the practice at this laboratory to develop the standard and unknown simultaneously in the same

crock, although not necessarily side-by-side on the same sheet as in the color comparison method. In this way the factors influencing spot size (diffusion effects, temperature, solvent composition, etc.) can be made uniform for a given determination. Particular care must be taken to apply the color-developing reagent uniformly and lightly, especially where the reagent used has a high water content. Spreading or streaking of the spots may result from too liberal application of the reagent. Subjective errors of judgment in the marking of the spot areas may be minimized by having the same person mark (with a sharp pencil) all of the areas in any given determination. Areas must, of course, be marked before the spots have faded, which in the case of many substances means immediately following color development. We have used a polar planimeter (Keuffel and Esser No. 4236) for measurement of the areas.

The minimum quantity of a substance which may be quantitatively estimated by this procedure is set by the size of the sample spot originally applied to the paper. Variations in the size of the originally applied spot will be reflected in the size of the spot resulting upon development of the chromatogram. For these reasons it is of utmost importance that the materials to be chromatographed be applied in small, uniform quantities. Applying the samples in five microliter increments (resulting in initial spots of about one centimeter diameter), makes the effective range about five to fifty micrograms for most substances.

Standard spots are usually run in quadruplicate at concentrations corresponding to 5, 10, 15, 20, 25, 30, 40, and 50 micrograms. Unknown spots are applied in such number and at such dilutions that at least four spots will fall in the range of 10 to 40 micrograms. A standard curve is constructed from the averaged areas of each standard dilution, and values corresponding to the areas of the unknown spots read from this curve.

The spot area method is, in large measure, complementary to the color comparison method. It is frequently useful where the latter cannot be used. Its lack of sensitivity to small quantities as compared to the color comparison method can often be put to advantage in avoiding large dilution of samples. Its most evident advantage is that of providing an empirical means for estimating the relative amounts of unidentified substances. Although considerably more time consuming than the color comparison method, if carefully performed, it is probably capable of greater accuracy.

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III. The Influence of Solvent Composition, Temperature and Some Other Factors on the Rf Values of Amino Acids in Paper Chromatography

by

B. JIRGENSONS*

Introduction

The purpose of this work was to obtain more knowledge about the fundamental dependencies of the Rf values on solvent composition, temperature, pH and the presence of salts. This knowledge might be of certain value for the improving of the results of practical work in the chromatographic determination of amino acids, as well as in obtaining more particular basic information as a background for a satisfactory theory of paper chromatography which might be elaborated in time. From the practical point of view it would be desirable if solvents other than those commonly employed (phenol, lutidine, collidine) were available for use.

Systematic research about the water-miscible solvents in the detection of amino acids by paper chromatography was done recently by Bentley and Whitehead (1). They used as solvents some aliphatic alcohols, acetone, furfuryl alcohol, tetrahydrofuran, and tetrahydrofurfuryl alcohol mixed with water. Some data about the influence of temperature on the Rf values can be found in the work of Consden, Gordon and Martin (2). According to these data the dependence of the Rf on temperature is complicated, especially for the widely used phenol solvent. Some new solvents have been used in the work presented in the following pages. The influence of the mentioned factors was investigated for some of these solvents. The most satisfactory spots and the best resolutions were realized through the use of some ternary solvent mixtures, containing water, propionic acid and such organic solvents as the butanols, ethylene glycol monobutyl ether or diethylene glycol monoethyl ether. Some important facts about the influence of temperature, pH and salt content have been found.

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Experimental

The experiments have been carried out according to the technique as proposed by Williams and Kirby (3). Each spot comprised 25 micrograms of amino acid; the cylindric stapled sheets were placed in very wide-mouthed brown glass bottles which were then tightly closed with screw caps. Each bottle contained 100 ml. of the solvent. The bottles were put in a thermostatically controlled water bath at definite, constant temperatures; the temperature was kept constant by using a cooling and heating unit with relay in a large bath with three stirrers. The sheets were dried and developed with ninhydrin. The Rf values were then measured in the proper manner by marking the centers of the spots and by measuring the distance of the solvent front and the distance of the center of the spot from the start line; the Rf is the ratio of the distance of the spot to the distance of the solvent front.

The results are presented in the following tables and figures

TABLE I

The Rf Values for Several Amino Acids Resolved with *tert*-Butanol-Water Mixtures at 30° C.

Vol.-%water	*10%*	*20%*	*30%*	*40%*	*50%*	*60%*
Glycine	.02[3]	.11[7]	.21[3]	.46[7]	.58[0]	.69[2]
Alanine	.04[7]	.18[5]	.30[0]	.53[6]	.63[6]	.76[1]
Aminobutyric acid	.10[0]	.24[2]	.36[0]	.53[5]	.68[0]	.79[5]
Valine	.13[7]	.31[9]	.44[4]	.63[9]	.71[0]	.80[5]
Leucine	.25[6]	.49[3]	.60[4]	.76[7]	.81[8]	.84[0]
Isoleucine	.25[8]	.42[7]	.58[0]	.70[0]	.77[5]	.85[0]
Norleucine	.29[5]	.51[0]	.64[0]	.75[0]	.81[0]	.86[0]
Phenylalanine	.14[0]	.40[2]	.53[4]	.71[4]	.75[6]	.79[6]
Serine	.02[6]	.12[4]	.23[9]	.48[0]	.59[2]	.71[4]
Glutamic acid	.00	.06	.17[0]	.36[8]	.60[2]	.75[0]
Lysine-HCl	.00	.02[5]	.09[6]	.20[0]	.29	
Methionine	.14[7]	.32[5]	.48[0]	.62[0]	.72	

TABLE II

The Rf Values for Several Amino Acids Resolved with Diethylene Glycol Monoethyl Ether-Water Solvent at 20° C.

Vol.-%water	*10*	*20*	*30*	*40*	*50*
Glycine	.25[6]	.36[6]	.48[4]	.62[2]	.68[3]
Alanine	.40[0]	.53[0]	.63[7]	.73[5]	.77[6]
Aminobutyric acid	.48[7]	.59[5]	.68[2]	.76[0]	.82[0]
Valine	.55[6]	.65[0]	.73[0]	.79[5]	.84[7]
Leucine	.63[2]	.68[8]	.74[2]	.81[5]	.85
Serine	.29[3]	.41[0]	.53[8]	.64[8]	.71
Glutamic acid	.33[0]	.57[5]	.60[0]	.71[8]	.80
Lysine-HCl	.09[8]	.22[6]	.27[3]	.26[8]	.28

TABLE III

The Rf Values for Several Amino Acids Resolved with Diacetone Alcohol-Water Mixtures at 30° C.

Vol.-%water	*10*	*20*	*30*	*40*	*50*
Glycine	.02[2]	.11[2]	.27[0]	.43[2]	.58
Alanine	.05[1]	.19[0]	.37[0]	.51[5]	.66
Aminobutyric acid	.08[5]	.25[0]	.43[6]	.57[5]	.70
Valine	.13[5]	.33[9]	.51[0]	.61[6]	.73
Leucine	.14[7]	.41[0]	.61[0]	.66[4]	.77
Serine	.01[8]	.11[8]	.28[8]	.45[0]	.60
Glutamic acid	.02[2]	.12[8]	.34[0]	.48	.64
Lysine-HCl	.00	.05[0]	.12[8]	.20	.23

TABLE IV

The Rf Values of Some Amino Acids Resolved with a Ternary Solvent Mixture of 15 vol. Water, 15 vol. Propionic Acid and 70 vol. Diethylene Glycol Monoethyl Ether; T=19° C.

Glycine	.36[0]	Aspartic acid	.39[3]
Serine	.37[8]	Glutamic acid	.62[0]
Alanine	.52[0]	Arginine-HCl	.40[8]
Aminobutyric acid	.59[7]	Histidine-HCl	.27[8]
Valine	.68[4]	Tyrosine (Na-salt)	.64[0]
Leucine	.74[0]	Tryptophan-HCl	.72[6]
Lysine-HCl	.38[7]	Threonine	.50[4]

TABLE V

The Rf Values for Some Amino Acids Resolved with a Ternary Solvent Mixture Containing 20 vol. Water, 20 vol. Propionic Acid and 60 vol. Ethylene Glycol Monobutyl Ether; T=8°, 17° or 28°

	8°	*17°*	*28°*
Glycine	.17[3]	.19[4]	.20[5]
Serine	.16[0]	.18[0]	.18[7]
Alanine	.26[0]	.27[6]	.28[2]
Valine	.42[5]	.44[8]	.46[8]
Leucine	.57[3]	.59[0]	.59[8]
Isoleucine	.53[6]	.56[0]	.57[6]
Threonine	.22[0]	.23[2]	.24[9]
Methionine	.46[1]	.47[4]	.50[6]
Aspartic acid			.18[0]
Glutamic acid	.20[4]	.22[8]	.24[7]
Lysine-HCl	.11[1]	.10[9]	.12[4]
Arginine-HCl			.14[7]
Histidine-HCl			.13[5]
Tyrosine (Na-salt)			.41[0]
Tryptophan-HCl	.47[8]	.49[0]	.52[0]
Phenylalanine			.58[0]

The most pronounced spots and the best separations were achieved with the solvents containing 30 volumes water and 70 volumes of the organic solvent. The reproducibility was good. Streaking of some amino acids takes place in the solvents with 10% water (e.g. leucine); lysine streaks at a higher water content of 40 to 60 vol-%. The differences in capillary ascent of sev-

eral amino acids with propionic acid-water solvent mixtures and ethylene glycol monobutyl ether-water mixtures are presented in the Figures 2 and 3. In the case of propionic acid the Rf values differ most when the water content is least.

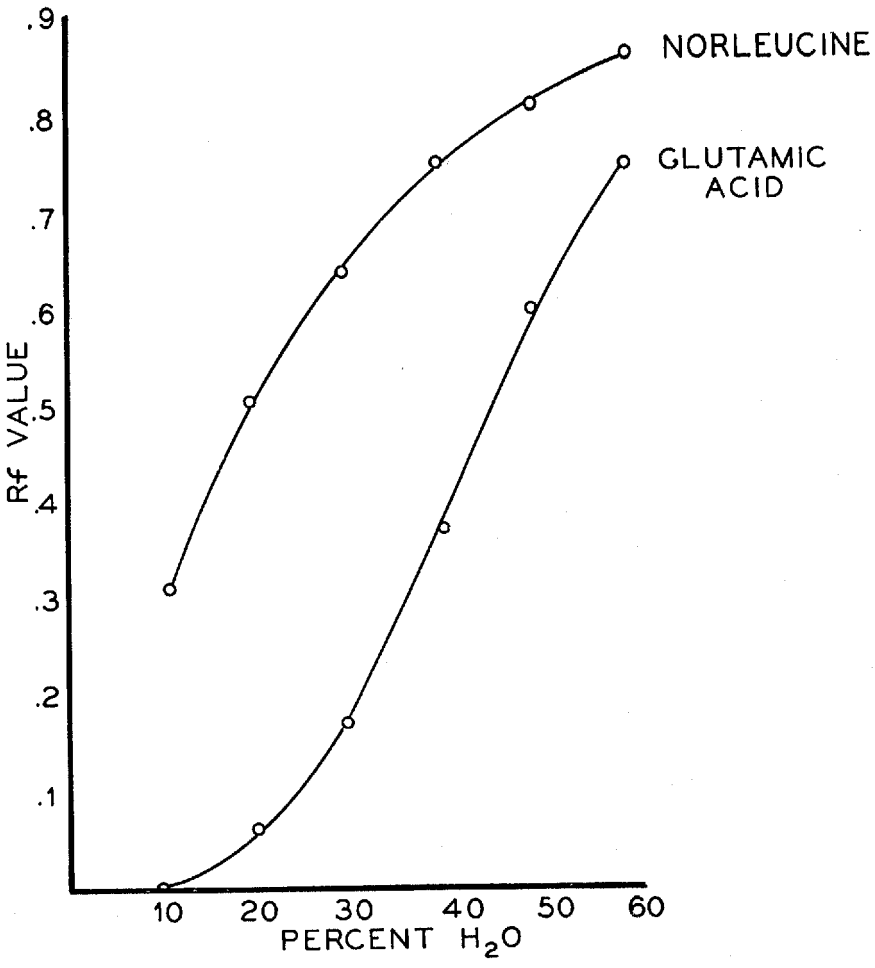

FIGURE 1

The dependence of Rf values on the water content of *tert*-butanol-water solvent mixtures.

The dependence of the Rf values on temperature was investigated mainly using as solvents the *n*-propanol-water systems. The temperature was kept constant within the limits of approximately one degree; the bottles containing the solvent and paper were

tightly closed. The results are presented in the Figures 4, 5 and 6. It was found that excellent spots and a good separation can be obtained by applying as solvents some ternary mixtures containing 15 to 30 volumes water, 15 to 30 volumes propionic acid, and the rest (up to 100) either ethylene glycol monobutyl ether or diethylene glycol monoethyl ether; instead of the latter two solvents, also some esters or alcohols may be used.

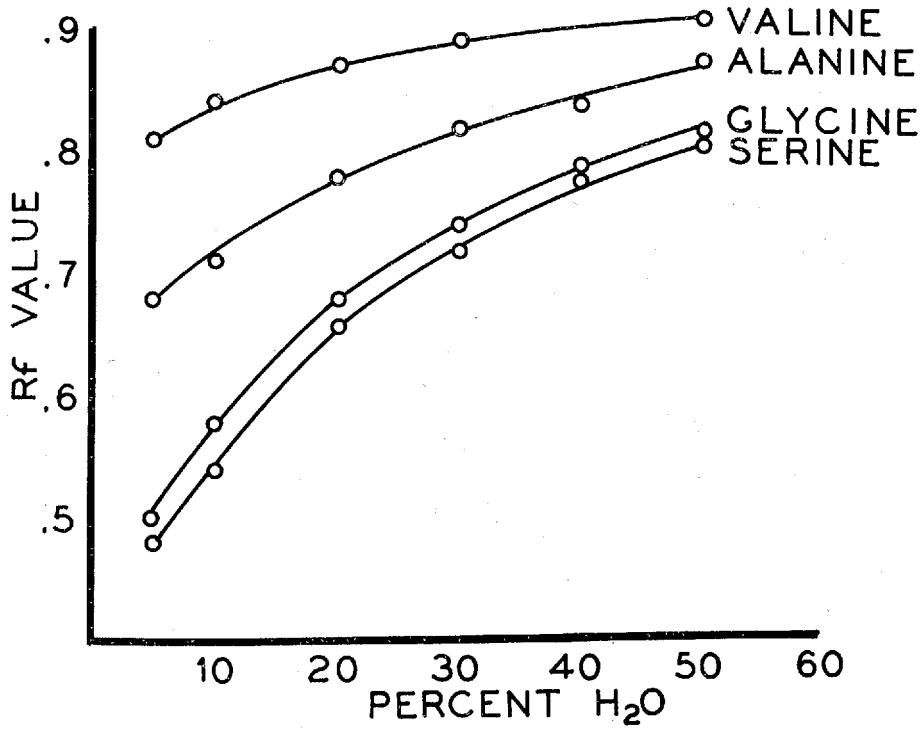

FIGURE 2

The dependence of the Rf values of several amino acids on the water content in propionic acid. T = 17° C.

Since it is impossible to dissolve some amino acids to a sufficient extent in pure water, making it necessary to add acid or alkali, it is important to know how the pH of the amino acid solution influences the Rf values. Some of the results are presented in Table VI.

It is quite evident that the Rf values depend on the pH of the solutions of the amino acids; especially in the cases of tyrosine and glutamic acid the differences are remarkable. The same was

observed if other neutral solvents were used. For instance with a ternary solvent mixture composed of 25 vol. water, 30 vol. *n*-propanol and 45 vol. *n*-butanol at pH=0.8 the Rf value of tyrosine was .480 but at pH=10.7 it was .377; the Rf values for glutamic acid at pH=1.5 and 9.2 were .231 and .085 respectively.

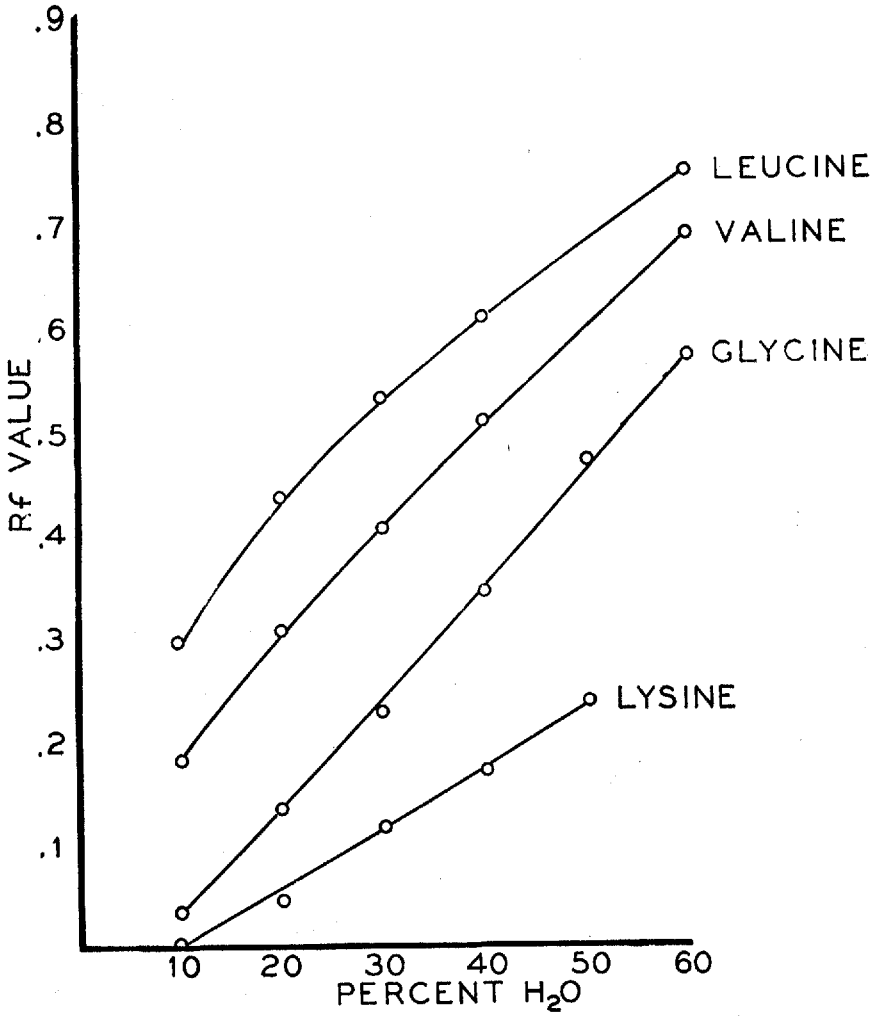

FIGURE 3

The dependence of the Rf values of several amino acids on the water content in ethylene glycol monobutyl ether. T = 18° C.

A quite different picture is revealed if the pH of a neutral *solvent* is changed by adding acid (HCl) or alkali (NaOH). Some of the

TABLE VI

The Rf Values as Depending on pH of the Solution. Solvent: 32 vol. Water with 8 vol. of *tert*-Butanol. T = 27° C.

	pH	*Rf*
Glycine	2.0	.32[5]
	7.1	.29[3]
	10.0	.28[2]
Serine	1.7	.32[5]
	6.5	.28[9]
	11.4	.29[5]
Tyrosine	0.8	.55[4]
	10.7	.44[6]
Tryptophan	1.45	.53[8]
	11.8	.49[2]
Glutamic acid	1.5	.43[3]
	3.0	.29[2]
	9.2	.17[8]
Lysine	1.4	.15[0]
	6.15	.13[3]
	11.0	.15[8]

results are presented in Figure 7. In a strongly acidic solvent of pH one to two, the Rf values are very high; the spots after developing are pink or purple. In a broad range between pH 4 and 11 the Rf values are constant.

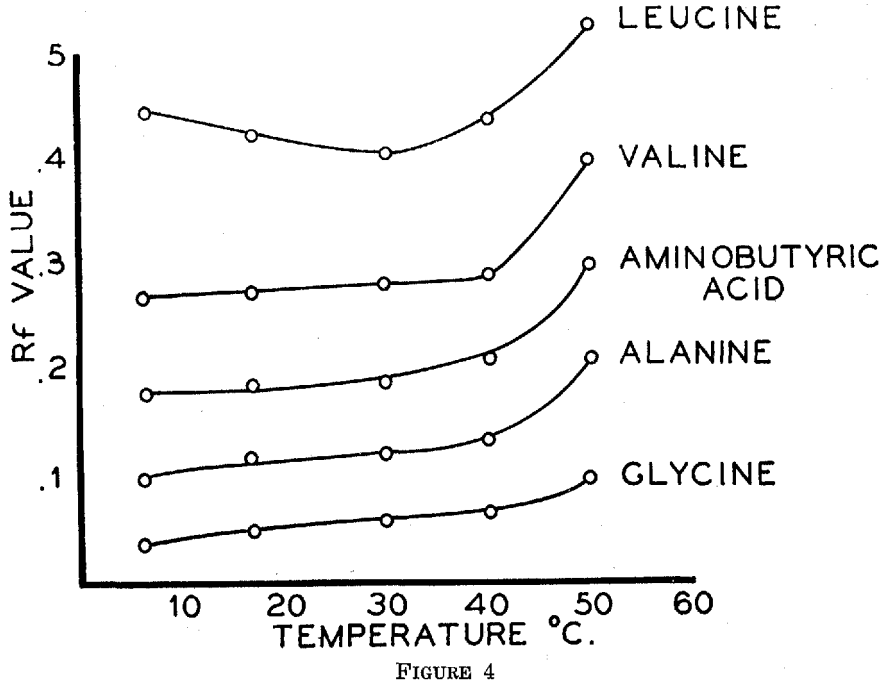

FIGURE 4

The dependence of the Rf values on temperature. Solvent: 10 vol. water + 90 vol. *n*-propanol.

The dependence of the Rf value on the salt concentration of the solvent was investigated mainly with the propanol-water and ethanol-water solvent mixtures. The latter were chosen because the ethanol-water systems, including those containing salts, have been widely used and it was hoped that from these data it would be possible to make some theoretical conclusions with respect to paper chromatography. Moreover, it might be expected that the salts would make the spots more distinct, perhaps preventing the streaking, or would change the Rf values in such a way as to improve the separations. Indeed, there is a definite influence of a neutral salt like sodium chloride on the Rf values as is shown in Table VII and Figure 8. It is noteworthy that sodium chloride completely prevents the streaking of lysine, histidine and arginine

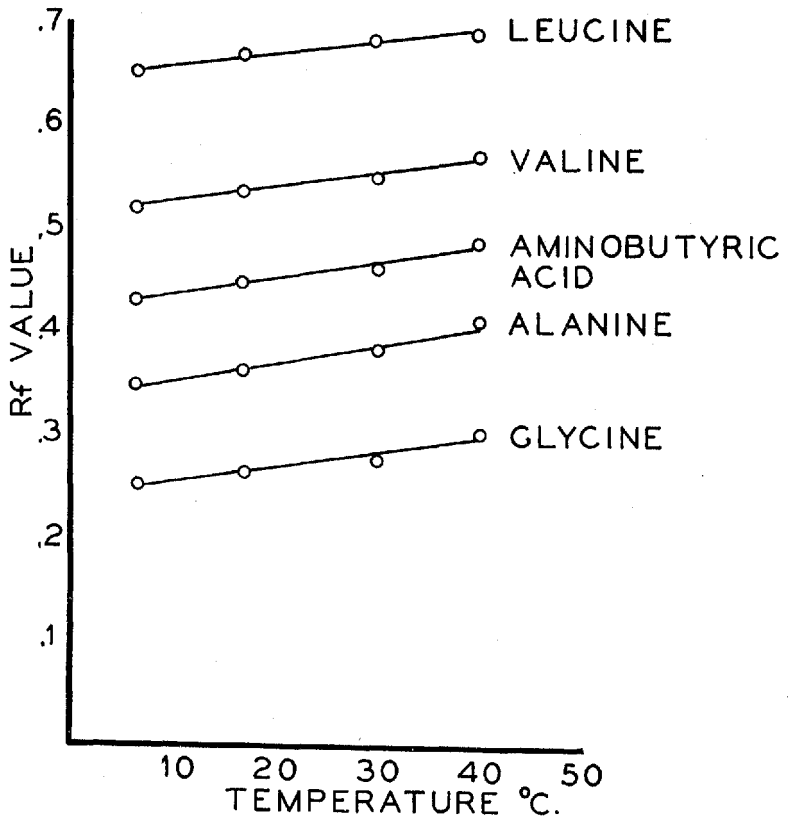

FIGURE 5

The dependence of the Rf values for several amino acids on temperature. Solvent: 30 vol. water + 70 vol. *n*-propanol.

but causes some streaking of aspartic and glutamic acid. The Rf values of most amino acids (most of which are acidic) decrease with increasing salt concentration, except lysine, arginine and

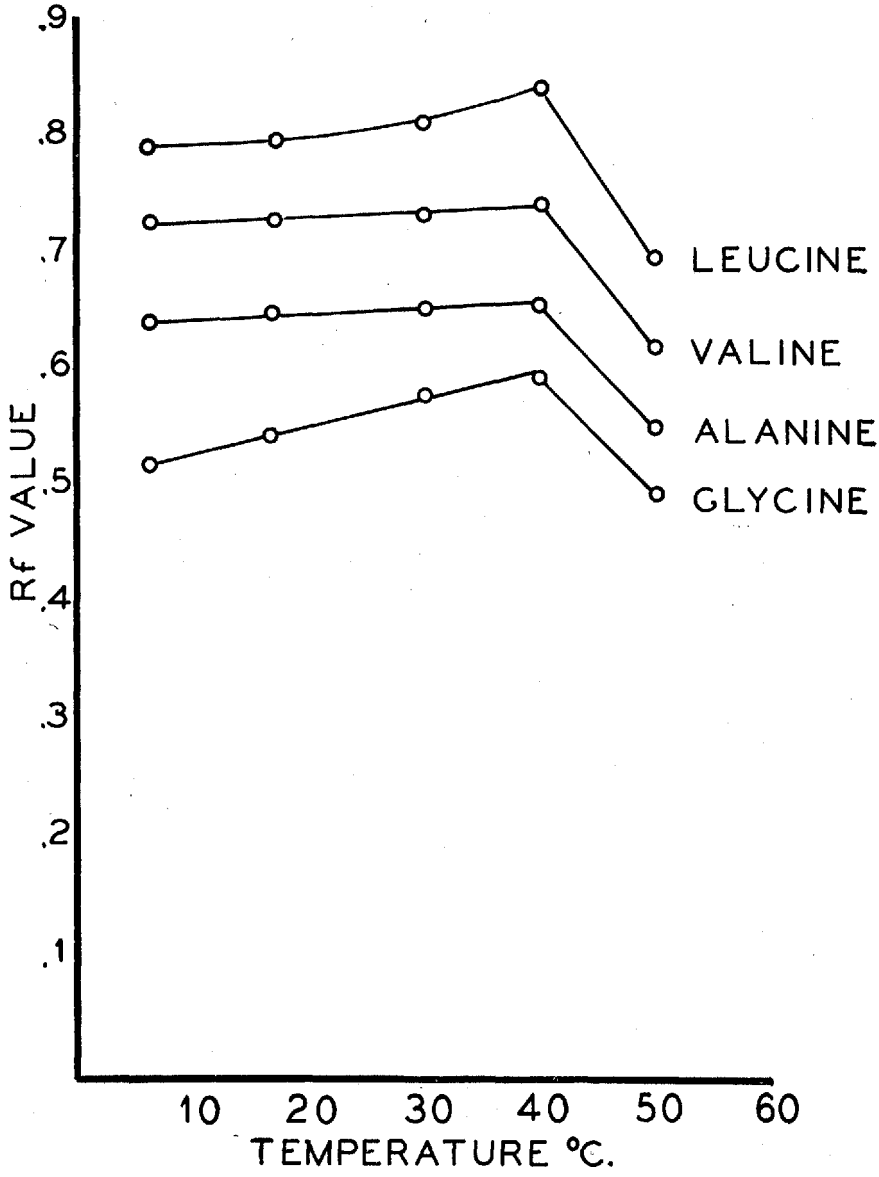

FIGURE 6

The dependence of the Rf values on temperature. Solvent: 50 vol. water + 50 vol. *n*-propanol.

histidine whose Rf values increase in the presence of salt in the solvent. The spots in the lower part of the sheets, after development with ninhydrin, appear pink or purple; the tyrosine, tryptophan and histidine spots are greyish.

TABLE VII

The Influence of Sodium Chloride on the Rf Values. Solvent: 66.5 vol. Ethanol + 33.5 vol. Water Saturated with NaCl. T=18°.

	Rf without NaCl	*Rf with NaCl*
Glycine	.39[6]	.38[6]
Serine	.41[2]	.42[9]
Alanine	.55[0]	.54[9]
Valine	.66[7]	.68[2]
Leucine	.69[5]	.70[0]
Glutamic acid	.47[8]	.36[6]
Lysine-HCl	.22[8]	.43[5]
Arginine-HCl	.22[1]	.46[9]
Histidine-HCl	.23[9]	.40[8]

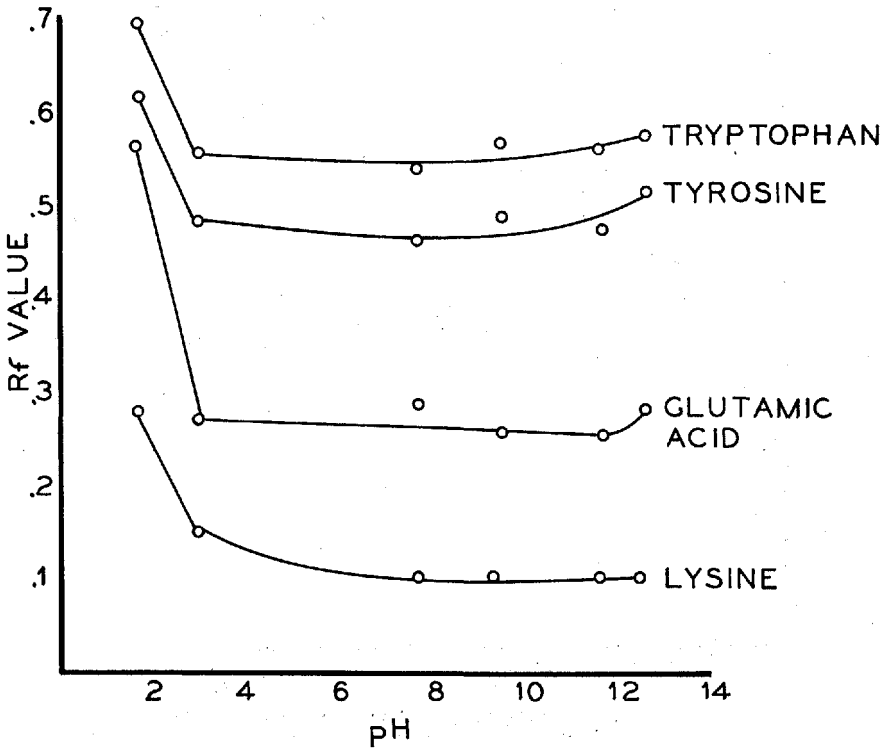

FIGURE 7

The dependence of the Rf values on pH of the solvent, composed of 25 vol. dil. HCl or NaOH + 75 vol. *n*-propanol. T = 28° C.

Water miscible solvents were used in this study because of the possibility of changing the ratios through wider limits. As Bentley and Whitehead recently proved (1) there are some water-miscible organic liquids (some furane derivatives, acetone, propanol) which in mixtures with 20 to 40% water give satisfactory spots and good separations. It was found in this work that quite

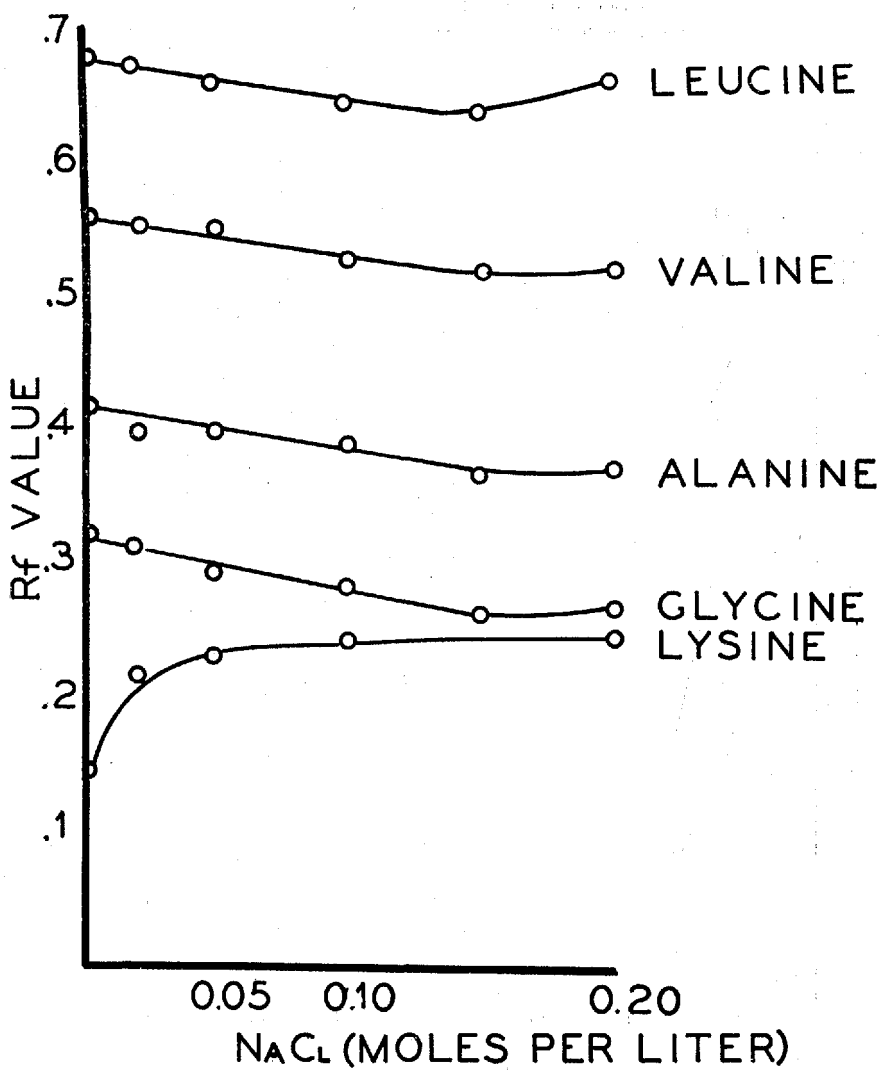

FIGURE 8

The dependence of the Rf values on the salt content of the solvent containing 32% water and 68% *n*-propanol. T = 17° C.

useful in this respect are also some glycol ethers, especially ethylene glycol monobutyl ether and diethylene glycol monoethyl ether. Again, the best spots and the best separation can be obtained using 20 to 30% water in the mixtures. Quite satisfactory are also the *tert*-butanol-water solvent mixtures. In the Figures 1 to 3 the Rf values are plotted as dependent on water content of the solvent, and these graphs show that there are several types of the curves obtained. In Figure 1 (for *tert*-butanol) it is shown that the Rf values of some amino acids (e.g. glutamic) increase very markedly with increasing water content, whereas the Rf values of leucine and its isomers change much less; consequently we obtain curves bent in opposite directions, i.e. a maximum separation or maximum difference in the Rf values at some intermediate water content (20 to 40%). The same type of curves are exhibited by the propanols, diacetone alcohol and some other solvents. A quite different curve system was obtained in the case of propionic acid (Figure 2) where the differences in Rf values diminish with increasing water content. In such cases the best separation is at the lowest water content used. However, in all the investigated cases, at a low water content of 5 to 10% water some of the amino acids streak. This applies also to the alcohols and glycol ethers. The latter give curve systems which are of somewhat intermediate type (Figure 3). This was one of the reasons which induced the author to try the ternary mixtures of water-propionic acid-glycol ether, and similar systems. Satisfactory spots and good separation were achieved by the use of 15 to 30% water, 15 to 30% propionic acid, and the rest ethylene glycol monobutyl ether or diethylene glycol monoethyl ether. The same satisfactory results were obtained with ternary solvents containing (a) liquids which are partially miscible with water such as *n*-butanol or ethyl succinate, (b) propionic acid or propanol, and (c) 15 to 20% water. The best separation of the difficulty separable glycine and serine was realized with a ternary solvent mixture containing 20 vol. water, 40 vol. propionic acid and 40 vol. ethylene glycol monobutyl ether.

The practical conclusion regarding the influence of the temperature is that the commonly occurring changes of room temperature have too little influence on the Rf values to distort the results materially, especially where adequate controls are included. However, for good reproducibility of the chromatograms it is advisable to keep the room temperature as uniform as possible. The reproducibility in work with carefully prepared solvents, uni-

form working conditions, and at thermostatically controlled constant temperature is good; in repeated experiments the Rf values then vary only within the limits of some two to five per cent. In such cases it is not unreasonable to calculate the values to the third decimal place; however, the third figure is not highly accurate and is so indicated in the Tables.

There is a definite influence of the pH of the amino acid on the Rf value, especially for tyrosine and glutamic (probably also aspartic) acid, if neutral solvents are used. The pH of the solvent too has a definite influence on the ascent. In the solvents containing organic acids the pH influence is present. It is important that the quality of the spots can be improved by changing the pH of the material and of the solvent.

Sodium chloride, if added to the solvent, prevents the streaking of the basic amino acids and considerably increases their Rf values; however, the Rf value of glutamic acid is decreased. Thus we have a convenient means of changing the Rf values in certain cases and of improving the definiteness of the spots also in this way. It would be desirable to investigate these problems more extensively, using other salts and other solvents. Another broad problem is the one involving the salt content of the amino acid solution to be chromatographed. There is comparatively little to be said about the theoretical conclusions. There must be a partition of the amino acids between the stationary phase of the water in the cellulose fibers and the mobile phase of the solvent, and the more water-rich the solvent the higher the amino acids ascend. However, that there is not exclusively a partition merely caused by the differences in solubilities, is now quite generally admitted. If this were the case it is difficult to understand why glycine and serine, whose solubilities are so different, have the same or nearly the same Rf values. According to Dunn and Ross (4) in 74.2% ethanol the solubility of glycine is 0.448 g. per 100 g. solvent, but that of serine is only 0.084 g. Also in pure water glycine is much more soluble than serine.

If partition only were involved there should be expected also a great influence of the salts on the partition of all amino acids. Actually, there is little influence of salt on the ascent of leucine. In alcohol-water mixtures the influence of the electrolytes on solubility is great; leucine is then dissolved or "salted in" as is also glycine and alanine. Therefore on the partition hypothesis the presence of salt in the alcohol-water solvent mixture should have a marked effect on the Rf values for these amino acids. This is

not the case; salt has a great influence only on such amino acids as lysine or glutamic acid.

Summary

The capillary ascent of amino acids on paper according to the method of Williams and Kirby was investigated and the dependence of the Rf values on solvent composition, temperature, pH of the amino acid and of the solvent, and on the salt content of the solvent was studied. It was found that:

1. The Rf values increase with increasing water content in a regular manner, and characteristic curves are obtained when the Rf values of the amino acids are plotted against the water content of different solvents. Tertiary butanol-water, propionic acid-water, and ethylene glycol monobutyl ether-water represent three different types of solvent mixtures.

2. Some ternary solvent mixtures containing 15 to 30% water, 15 to 40% propionic acid, and the rest ethylene glycol monobutyl ether or diethylene glycol monoethyl ether yield excellent spots and good separation.

3. The Rf values usually increase slightly with increasing temperature. For the solvent composed of 30 vol. water and 70 vol. *n*-propanol, the increase within the limits of 6° to 40° C. is linear.

4. The Rf values are influenced by the pH of the amino acid solution applied on the sheet, especially in the cases of tyrosine and glutamic acid.

5. The Rf values depend also on the pH of the solvent. The Rf value increases in strong acid and strong alkaline solutions, but is practically constant within the limits of pH 4-11.

6. The Rf values depend on the salt concentration of the solvent; there is a remarkable decrease of Rf values for glutamic acid with increased salt concentration. On the contrary, the Rf values of lysine, arginine and histidine increase greatly in the presence of salt, and the latter prevents streaking of these amino acids in the solvent tested.

Acknowledgment

The author is cordially grateful to Dr. Roger J. Williams, Director of the Biochemical Institute of The University of Texas, for the facilities to carry out this research. Many thanks also to Dr. Norman Hackerman for his interest toward this work.

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IV. Quantitative Study of Urinary and Salivary Amino Acids Using Paper Chromatography

by

HELEN KIRBY BERRY AND LOUISE CAIN

Of all the chromatographic procedures which have been developed, perhaps the most extensively used method has been the quantitative determination of the amino acids. At this laboratory the urinary excretion of these substances has been studied for a considerable number of subjects and in some cases for considerable periods of time. In addition, many saliva specimens have been analyzed. The results of these studies are reported principally in other portions of this bulletin.

With a mixture containing as many amino acids as urine frequently contains, the use of ordinary one-dimensional chromatograms which have been previously described is frequently impractical. However, after the preliminary use of the two-dimensional chromatograms, the one-dimensional method can, with selected individuals and for certain amino acids, be effectively employed.

One-Dimensional Chromatograms

Although the specific details of the one-dimensional chromatographic procedure have been previously published (1), certain of the essential features will be repeated here. The method is useful in the determination of urinary glycine, lysine, alanine, leucine, valine, glutamic acid, and aspartic acid. However, when the urine sample in question contains more than very small amounts of asparagine and taurine, glycine cannot be determined using one-dimensional technique. Similarly, where glutamine, β-alanine, tyrosine, or citrulline are present in considerable amounts, alanine cannot be determined. Threonine and arginine likewise may interfere with the determination of lysine. Hence, a preliminary test of the sample using two-dimensional chromatograms should always be run to determine the abundance of these substances relative to the amounts of the amino acids to be determined. When the combined interfering substances are more than about 10% of the quantities of glycine, alanine and lysine present respectively, recourse must be made to the two-dimensional technique.

The essential features of this procedure are similar to the other color comparison methods which have been previously described. The details concerning ranges and application of standard and sample can be found elsewhere (p. 23). The chromatograms are run in the phenol solvent (p. 25) and dried at room temperature. Color is developed using the ninhydrin reagent described on p. 27 followed by heating for five minutes at 100° C. The matching of spots and the calculation of amounts present are carried out in the same manner as has been previously described.

The advantages of the one-dimensional over the two dimensional method are twofold: the saving of time in the preparation and development of chromatograms, and the facility and increased accuracy in the matching of spots in close proximity. However, in many individual cases the presence of the interfering substances makes the one-dimensional procedure impractical.

Two-Dimensional Chromatograms

Chromatographic procedure.—The procedure followed in making two-dimensional chromatograms is essentially that of Polson (2); however, there are enough modifications of the method to warrant a more detailed description of the technique.

The special problems involved in the preparation of such chromatograms have to do chiefly with the choice and application of the second solvent and with the complications which arise in the quantitative interpretation of the chromatograms.

In this laboratory, the phenol solvent (p. 25) has been routinely employed for the initial development, and the 2,6-lutidine solvent for the other dimension.* After the initial development with the phenol solvent, the sheets must be dried thoroughly before using the lutidine solvent. The sheets run in lutidine as a second solvent show a characteristic brown front at an Rf of .75-.90, apparently due to a residue from the phenol solvent on the paper. The Rf values, based on the total ascension height of the lutidine solvent, are always lower in this case than those obtained from a one-dimensional chromatogram developed with the lutidine solvent alone. If, however, the Rf values are calculated on the two-dimensional chromatogram using the brown

*Most other workers involved in the analysis of amino acid mixtures employ routinely for the second dimension either a fraction of the mixed collidines or 2,4,6-collidine. We have avoided the mixture because of the variable results it yields and the pure 2,4,6-collidine because of the expense of using it in an extensive series of analyses.

front as the effective lutidine solvent height, excellent agreement is observed with the Rf values obtained with the one-dimensional chromatograms. This effect can be minimized by discarding the phenol solvent at the first appearance of any brown discoloration in the solvent, and in any case within 48 hours after its preparation. The lutidine solvent should also be discarded at the first appearance of its discoloration; otherwise colors and intensities of the amino acid spots after development with ninhydrin will be distorted and difficult to compare. Since strong acid vapors may cause atypical red colors rather than purple with certain amino acids, contact with such vapors must be avoided during the development of the chromatograms.

The chromatograms are prepared using 9x11″ sheets. On the individual sheet the single standard (or sample) spot is placed one inch from a corner. The general practice at this laboratory has been to prepare a series of two-dimensional chromatograms containing 0.3, 0.6, 0.9, 1.2, 1.5, 2.0, 2.5, and 3.0 micrograms respectively of each of the following amino acids: aspartic acid, glutamic acid, serine, glycine, lysine, alanine, leucine, valine, taurine, threonine, citrulline*, and methionine sulfoxide. Volumes of urine equivalent to 20 and 40 micrograms of creatinine respectively were used to prepare two-dimensional chromatograms for comparison with the standard chromatograms. Not all the amino acids in the standard mixtures appear in each urine sample, since the pattern of amino acids excreted by each individual is characteristic. The practice of superimposing small amounts of urine on the standards has not been followed here since it is both impractical and unnecessary.

The series of standards and sample chromatograms should be prepared and developed at the same time and in the same manner. It is particularly important that the drying treatment to remove the first (phenol) solvent be identical for standard and sample sheets.

Quantitative Estimation.—The quantitative estimation of the amino acids on two-dimensional chromatograms, while the same in principle, is somewhat more tedious and subject to greater errors than is the case with one-dimensional chromatograms. In order to make color comparisons as exact as possible, the sample

*Since citrulline, β-alanine, and glutamine are not separable using the two solvents employed here, the spot on the sample sheets corresponding to the citrulline spot on the standard sheets may also contain β-alanine and glutamine.

and standard sheets are viewed side by side on a lighted X-ray viewing screen. Inasmuch as the standards and samples are on separate sheets, the chance for errors arising from difference in treatment is somewhat increased, and the ability to make the exact comparisons possible in the alternate spot arrangement of the one-dimensional chromatograms is decreased. In addition, the rather wide intervals between the different standard sheets make the absolute error of a given determination larger. However, for the purpose of comparing individual amino acid excretion patterns the technique gives results of sufficient accuracy.

Fig. 1 is a composite diagram of the spots observed on two-dimensional chromatograms of urine and saliva. All of these spots were not observed in any single sample, and some few of them have not, as yet, been identified with any known substance. The identifications given in Fig. 1 were based on comparisons with standard chromatograms of known substances and the addition of "marker" amino acids to the urine samples. Comparisons with amino acid "maps" of other experimenters have also been made (3). *Spot 7* is the position taken by citrulline, glutamine and β-alanine and we have not found it possible to distinguish between them since each possesses practically identical Rf values in both phenol and lutidine. *Spot 16* remains unidentified. Its position corresponds to that of ethanolamine on Dent's (3) map, but is unaffected by addition of ammonia or acid to the atmosphere during phenol development, whereas ethanolamine is shifted markedly under these conditions. *Spot 17* has been provisionally identified as methyl histidine. This amino acid was not available to us, but the color and position of the spot corresponds to that of methyl histidine as described by Dent (3). *Spots 21* and *22* are unidentified. *Spot 27* may correspond to the "nephrosis peptide" of Dent (3).

A great deal of effort has been spent in an attempt to identify the substance responsible for *Spot 26*. This is the principal amino acid in all saliva samples investigated. The paper chromatographic behavior of this substance as compared with known substances of similar behavior is summarized in Table I. The Rf values recorded were all obtained in the presence of small amounts of saliva to minimize the effect of interfering substances. For a considerable time it was thought that the substance appearing at *Spot 26* might be γ-amino butyric acid, but the behavior of the two substances in solvents containing acetic or isobutyric acid is different.

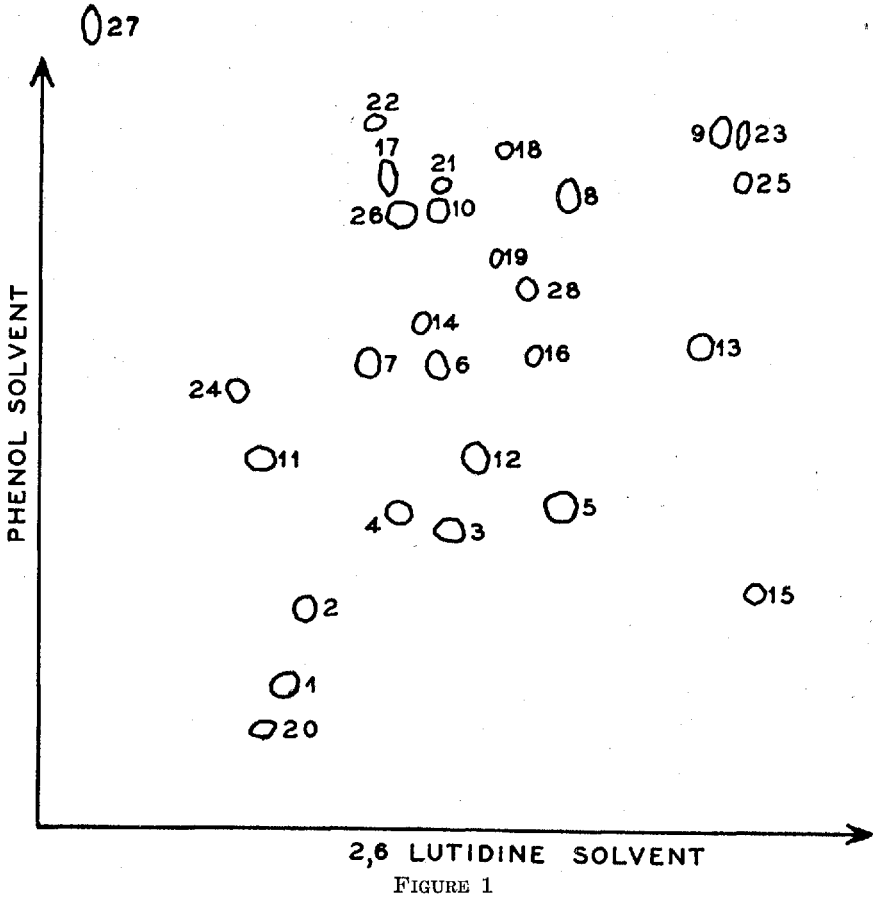

FIGURE 1

Composite Diagram of Spots Observed on Two-Dimensional Chromatograms of Urine and Saliva

Spot No.		Spot No.	
1	aspartic acid (blue)	16	unidentified (purple)
2	glutamic acid (purple)	17	methyl histidine (?) (blue-green)
3	serine (purple)	18	proline (yellow)
4	glycine (purple)	19	α-amino butyric acid (purple)
5	taurine (purple)	20	cystine (brown)
6	alanine (purple)	21	unidentified (purple)
7	citrulline and/or glutamine and /or β-alanine (purple)	22	unidentified (purple)
8	valine (purple)	23	phenyl alanine (blue)
9	leucine (purple)	24	arginine (purple)
10	methionine sulfoxide*	25	tryptophan (brown-purple)
11	lysine (purple)	26	unidentified salivary constituent-(position of γ-amino butyric acid) (see text) (purple)
12	threonine (purple)	27	"nephrosis peptide"?
13	tyrosine (blue)	28	methionine sulfone
14	histidine (brown-purple)		
15	cysteic acid (blue)		

*Since the material for this Bulletin was prepared it has become probable that the substance originally identified by Dent as methionine sulfoxide is actually β-aminoisobutyric acid. Nature, **167**, 307 (1951).

The possibility that this substance might be a peptide was considered and rejected on the basis of its presence in chromatograms run on acid hydrolyzed saliva. A pooled saliva sample was chromatogrammed, (a) without further treatment, (b) after hydrolysis with hydrochloric acid, (c) after precipitation of proteins with ethanol, and (d) after precipitation of proteins, followed by hydrolysis. None of these treatments appreciably affected Spot 26.

TABLE I

Comparison of Rf Values of Unidentified Substance in Saliva (Spot 26) with Known Amino Acids Suspected of being Identical

	*Rf Values in the Following Solvents**						
Amino Acid	*Phen*	*BuAc*	*BuEt*	*But*	*Lut*	*IsoBu*	*Py*†
α-amino *n*-butyric	.76	.41	.37	.19	.21	.56	.37
α-amino isobutyric	.78	.44	.37	.20	.19	.56	.42
γ-amino butyric	.85	.44	.22	.09	.10	.53	.22
leucine	.89	.67	.63	.49	.40	.76	.57
methionine	.83	.46	.46	.29	.34	.67	.49
methionine sulfone	.66	.16	.24	.06	.21	.38	.33
methionine sulfoxide	.85	.16	.21	.06	.10	.44	.21
pantonine	.81	.42	.39	.21	.24	.60	.44
phenylalanine	.89	.55	.59	.42	.40	.79	.51
valine	.83	.55	.46	.30	.28	.69	.47
"Spot 26"	.81	.54	.22	.07	.10	.70	.23

*A complete description of the solvents employed will be found on p. 25.
†This solvent was composed of 80% pyridine and 20% water.

The methods here outlined have found extensive application in our studies and the results of analysis by these methods will form an important part of the data presented in other parts of this bulletin.

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V. The Quantitative Determination of Urinary Histidine Using Paper Chromatography

by

LOUISE CAIN AND HELEN KIRBY BERRY

Considerable interest has been shown in the study of the urinary excretion of histidine, both in connection with its reported variation during the menstrual cycle and with its attempted use as a specific test for pregnancy. Boxer and Kapeller-Adler (1) demonstrated that the excretion of histidine was greatest about 14 days before the beginning of the menstrual cycle, corresponding to the ovulatory phase. However, Chattaway (2) was unable by testing urine samples at seven-day intervals to demonstrate any appreciable difference in histidine excretion during the menstrual period. The marked increase of histidine excretion fairly early in pregnancy has suggested its use as a diagnostic test (3, 4, 5).

The standard colorimetric tests suggested as a basis for the determination of histidine in urine are subject to uncertainty as a result of the lack of specificity of the reagents. The two most common reactions of histidine which are used for its determination are the bromination reaction described by Knoop (6) and subsequently modified by several investigators (7, 8), and the diazotized sulfanilic acid reagent of Koessler and Hanke (9). Attempts in our laboratory to use the bromination reaction as modified by Chattaway (8) to determine urinary histidine were unsuccessful in that no adequate recoveries could be obtained. We have attempted to use both of these color developing reagents for the determination of histidine in our usual paper chromatograms. In most urine samples there were several substances other than histidine which gave the same color reaction and caused interference by migrating to the same position. Consequently different solvents were explored.

Experimental

Solvent Mixture.—Although a number of different solvent mixtures were used in developing the procedure, all but one had serious disadvantages. In most of the solvents tested the histidine either did not migrate appreciably or it split to form two spots in the presence of urinary salts. However, using a solvent mixture consisting of 80 ml. of *n*-butanol, 20 ml. of ethanol, and 40 ml. of

2N hydrochloric acid, histidine migrates as a single spot to a position with an Rf of .20, free from interference by the substances which disturb the colorimetric procedure.

Reagent.—Both the bromine reagent and the diazotized sulfanilic acid reagent were employed in developing the quantitative procedure, but the latter, in addition to being more sensitive, was found to give more consistent and reproducible results. As little as 0.20 micrograms of histidine could be detected using this reagent, while 5-10 micrograms were required when the bromination reaction was carried out. The details for preparing and using the diazotized sulfanilic acid reagent may be found on p. 30. The exclusion of the vapors of phenol, pyridine, lutidines and collidines is essential to prevent interference with development.

Procedure.—The chromatograms were prepared using sheets of filter paper 5½ inches in height. Pure histidine in a standard solution was added in 5 microliter increments to alternate spots ¾ inch apart so as to have the following amounts of histidine present: 0.25, 0.50, 0.75, 1.0, 1.5, and 2.0 micrograms. At this stage a preliminary test was usually carried out by placing 5.0 microliters of the unknown urine on a small strip of filter paper and allowing the solvent to ascend about one inch above the applied spot. The strip was dried and developed with the diazotized sulfanilic acid reagent. If the preliminary test showed a distinct pink to red-orange spot, as the majority of urine samples did, 5 microliters of the urine was placed on the positions alternating with the standard histidine spots. Finally, the urine was diluted 1:2, and 5 microliters of the dilution was superimposed on the standard histidine spots to correct for the distorting effects of urinary constituents. The effective color range of the test is from a light pink to a conspicuous red-orange, and the unknown and standard were matched as described on page 50. Where the urine was too dilute to show a distinct reaction using 5 microliters, 10 to 30 microliters of urine were added to the sheet containing the standard histidine, and 5 microliters of undiluted urine was superimposed upon the standard. Wherever such relatively large quantities of urine were necessary, chromatograms 11 inches in height were employed to avoid interference and distortion. When the urine was relatively concentrated in histidine, 5 microliters of a 1:2 dilution was used and a 1:4 dilution was placed on the standard spots. The sheets were dried for 30 minutes at room temperature before spraying with the diazotized sulfanilic acid.

Results.—In Table I are given results of recovery experiments in which histidine was added to urine.

TABLE I

Recovery from Urine of Added Histidine

	Histidine Added (mg./ml.)	Histidine Found (mg./ml.)	Histidine Recovered (mg./ml)	Per Cent Recovery
Urine Sample 1	0.0	0.30	0	
	1.00	1.27	0.97	97
Urine Sample 2	0.0	0.45	0	
	0.65	1.16	0.71	109
Urine Sample 3	0.0	0.46	0	
	0.50	0.95	0.49	98
Urine Sample 4	0.0	0.30	0	
	0.80	1.12	0.82	103
Urine Sample 5	0.0	0.30	0	
	0.20	0.52	0.22	110

Summary.—A colorimetric procedure for the quantitative determination of histidine in urine employing paper chromatography is described. A special solvent mixture is used and diazotized sulfanilic acid is used for color development. The average recovery of histidine added to urine was 103%.

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VI. The Quantitative Determination of Creatinine in Urine Using Paper Chromatography

by

HELEN KIRBY BERRY AND LOUISE CAIN

Creatinine, which has been used in all of the subsequent studies on individual metabolic patterns as a basis for correcting for urine concentration (p. 152), has been routinely determined using a colorimetric procedure (1). However, where the urine samples available from young infants and from rats and mice were small (as little as a fraction of a milliliter in some cases), it was necessary to develop a technique which would require much smaller quantities than were needed for the colorimetric procedure.

Experimental

Solvent mixtures.—The solvent mixture used in the determination was composed of 80 ml. of *n*-butanol, 20 ml. of ethanol, and 20 ml. of distilled water. This solvent may be made up as a stock solution and aliquots used for two to three determinations before replacing with a fresh aliquot.

Reagent.—The reagent used for color development was the alkaline picric acid reagent described on p. 31.

Procedure.—Chromatograms for the determination of creatinine were prepared on sheets of filter paper 3 x 11 inches. Experience has shown that a solvent ascension of three inches is ample to remove distorting and interfering substances in this case. Pure creatinine in a standard solution was added to alternate spots 1½ inches apart so that the spots contained 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, 1.75, and 2.00 micrograms. On the positions alternating with the standard spots was placed 5 microliters of a 1:2 dilution of the sample of urine. Finally 5 microliters of a 1:4 dilution of the urine was superimposed upon the standard creatinine spots. After resolution and color development the creatinine content of the urine was calculated as described on p. 52. The Rf value of creatinine in the solvent used is .30. The color shade of the spots did not vary over the effective range, but the intensity of the color showed a considerable variation. Occasionally with concentrated

urines it was necessary to employ a 1:4 dilution as the unknown and to place a 1:8 dilution on the standard creatinine spots.

Results.—The results obtainable using the paper chromatographic procedure are compared with the results obtained from the standard colorimetric technique in Table I. Note that while the absolute errors are approximately the same, the relative error may be rather high when samples are dilute.

TABLE I

Comparison of Creatinine Determination by a Standard Colorimetric Procedure and by Paper Chromatography

Sample No.	*Creatinine by Colorimetric (mg./ml.)*	*Creatinine by Paper Chromatography (mg./ml.)*	*Percent Difference*
1	1.66	1.6	— 3.7
2	2.32	2.4	+ 3.4
3	1.56	1.6	+ 2.6
4	.48	.50	+ .4
5	.32	.25	—22.
6	.37	.40	+ 8.1
7	.35	.35	0.

Summary.—A paper chromatographic method for the quantitative determination of creatinine in very small amounts of urine is described. Alkaline picric acid is used for color development. Comparison of the results obtained by this method to the results obtained using the standard colorimetric technique are presented.

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VII. The Quantitative Determination of Creatine in Urine Using Paper Chromatography

by

LOUISE CAIN

The determination of creatine by the standard colorimetric method (1) is tedious and time consuming. A relatively simple method for the determination of creatine in urine has been developed using paper chromatography and is here presented. This method is especially useful when the quantity of the urine sample is limited and a large number of samples are to be analyzed.

Experimental

Screening Procedure.—Since creatine appears only infrequently in the urine, it is desirable to make a rapid screening of a large number of samples to ascertain its presence. For this purpose filter paper sheets eleven inches high are used, and samples of urine containing 50 μg. of creatinine (previously determined) are placed in the usual manner (p. 23) one inch from the bottom of the sheet and one inch apart. The samples are then resolved by allowing a solvent mixture composed of 80 ml. n-butanol, 20 ml. ethanol, and 20 ml. distilled water to reach the top of the sheet. The sheets are thoroughly dried, sprayed with a warm aqueous solution of 1.3% picric acid, and then heated for one hour at 110° C. in order to convert the creatine to creatinine. Finally the sheet is sprayed with a 1 *N* sodium hydroxide solution. If creatine was present in the original sample, the characteristic orange color of creatinine on a yellow background appears at an Rf value of .10. Since creatinine moves in this solvent to an Rf value of .25, there is no likelihood that the two substances will be confused. Using this butanol-ethanol-water mixture as a screening solvent has the added advantage that much valuable information can be gained concerning other substances which react with picrate and which migrate near the solvent front in such a solvent as lutidine.

Quantitative Determination.—Creatine, when resolved in the butanol-ethanol-water mixture, is sometimes distorted by an interfering substance also appearing in urine. Hence, it is desirable that samples which have been shown by the screening procedure to contain creatine be chromatographed using a mixture of 65 ml.

lutidine and 35 ml. water as the resolving solvent. A sheet of filter paper 5½ inches high is sufficient for this more precise determination. On alternate positions are placed 5 μl. quantities of standard solutions containing 3.0, 5.0, 8.0, 10.0, 12.5, and 15.0 μg. of creatine respectively. On the remaining positions are placed 10, 10, 15, 20, and 25 μl. quantities of urine. Finally, 5 μl. of urine is superimposed upon each spot of the standard solution. These volumes of urine may be changed depending on the concentration of creatine estimated to be present by the screening procedure. After resolution and color development as above, the concentration of creatine can be determined by matching the appropriate spot from a urine sample with the corresponding standard spot. The Rf value of creatine in this solvent is .27 while that of creatinine is .56.

In Table I are summarized the results of some recoveries of creatine as determined by this method. Creatine was added in the amounts listed to samples of urine which originally contained an insufficient amount of creatine to be detected by this method. It is probable, however, that in some cases small amounts were pressent. These minute amounts may account for the large error obtained when low concentrations of creatine were added.

TABLE I

Recovery from Urine of Added Creatine

Urine Sample	*Added Creatine (mg./ml.)*	*Creatine (mg./ml.)*	*Per Cent Recovery*
1	0.94	1.0	106
2	1.33	1.0	75
3	0.59	0.50	85
4	1.05	0.91	86
5	0.78	0.80	102
6	0.19	0.33	200
7	1.67	1.60	96
8	0.30	0.60	200
9	1.22	1.0	82

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VIII. The Quantitative Estimation of Uric Acid by Paper Chromatographic Methods, with Applications to Human Urine and Saliva

by

HELEN KIRBY BERRY

Uric acid is the principal end-product of purine metabolism in humans, and its concentration in the urine is known to vary widely with the purine content of the diet. Dent has studied the migration of uric acid on paper chromatograms using phenol and collidine as solvents and 1% ammoniacal silver nitrate solution as a color developing reagent (1).

Experimental

Selection of color reagent and solvent.—A solution 0.05 *N* in $AgNO_3$ and 2.5 *N* in NH_4OH was employed as the color developing reagent. Chromatograms were dipped in this solution, the uric acid spot developing as a black area within two to three minutes. The following solvent mixtures were investigated: (a) 65 ml. of 2,6-lutidine and 35 ml. of water; (b) 80 ml. of *n*-butanol, 20 ml. of ethanol, and 20 ml. of water; and (c) 100 g. of phenol and 20 ml. of 10% sodium citrate solution (2). Using 2,6-lutidine, uric acid appears as a well defined spot at Rf .31. With the butanol-ethanol solvent, uric acid forms a streak. In phenol uric acid migrates to an Rf value of .35, but the phenol causes almost immediate reduction of the silver nitrate applied as a color developer. 2,6-Lutidine was selected as the most satisfactory solvent.

Both the position of uric acid on the chromatogram and the rapid reduction of ammoniacal silver nitrate are very specific. The uric acid need be allowed to migrate only far enough to prevent interference from a substance, probably sodium ion, which does not move in lutidine, but which causes reduction of silver nitrate rather more slowly than does uric acid.

Chromatographic assay procedure.—The general techniques employed in preparing and developing the paper chromatograms have been described elsewhere (2, 3). Because of the specificity of the procedure only a short migration is necessary, and sheets 14 x 2½ inches have been employed. These provide 18 positions for application of sample and standards at ¾ inch intervals,

½ inch from the bottom of the sheet. About 20 minutes is required for the solvent to ascend to within ¼ inch of the top of the sheet.

The spot comparison method, described in detail elsewhere in this bulletin (2), was employed for quantitative estimation. Spots developed from five microliters of 1:2, 1:4, and 1:8 dilutions of urine were compared with adjacent spots developed from five microliters of 1:4, 1:8, and 1:16 dilutions of urine plus added uric acid. The nine standard spots on a sheet were made up to correspond to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, and 1.2 mg. uric acid per ml. urine. After determining the approximate uric acid content of a urine sample, if greater accuracy is desired, a second sheet can be run with smaller intervals between the amounts in the standard spots.

The sheets were dried in air for thirty minutes before treatment with the ammoniacal silver nitrate solution. Spots may be compared while the sheet is still wet, or it may be allowed to dry. The background of the sheet turns brown after several hours, unless excess silver nitrate is removed by washing.

Results

Recovery experiments.—The results of recovery experiments on two urine samples are shown in Table I. The recoveries varied from 100-133% with an average error of 17%. Each determination was made using a single chromatogram as described above. More precise values could no doubt have been obtained by employing a second chromatogram with a narrower range of standards.

TABLE I

Recovery of Added Uric Acid from Urine

	Uric Acid Added (mg./ml.)	*Uric Acid Found (mg./ml.)*	*Uric Acid Recovered (mg./ml.)*	*Percent Recovery*
Sample 1	0.0	0.6	0.0	
	0.086	0.7	0.1	116
Sample 2	0.0	0.13	0.0	
	0.1	0.23	0.1	100
	0.2	0.38	0.25	125
	0.3	0.53	0.40	133
	0.4	0.58	0.45	112
	0.5	0.70	0.57	114

Uric acid content of male urines.—Morning urine samples were collected from twelve men for five days a week over a four week period. The samples from each subject for each week were pooled on the basis of a constant creatinine content. Chromatograms were

run in duplicate, and when the variation between duplicates was greater than 20%, the determination was repeated. The results are shown in Table II. Values for uric acid are recorded as mg. of uric acid per mg. of creatinine, in order that the values for the various subjects may be compared on a basis more nearly independent of urine volume. Creatinine was determined by a standard colorimetric method (4). Inspection of the data reveals a greater variation in the excretion of a given individual from week to week, than in the average excretion rates of the different individuals. These data are in line with the known variation of uric acid excretion with the diet.

TABLE II

Urinary Uric Acid/Creatinine Ratios for Twelve Males

Subject	*First Week*	*Second Week*	*Third Week*	*Fourth Week*	*Average*
1	.37	.15	.29	.27	.27
2	.49	.41	.06	.31	.32
3	.66	.35	.13	.07	.31
4	.35	.41	—	.03	.26
5	.30	.23	.47	.33	.33
6	.24	.37	.23	.04	.22
7	.52	.22	.21	.11	.27
8	.39	.20	.08	.40	.27
9	.73	.55	.27	.13	.42
10	.55	.24	.31	.24	.33
11	.42	.34	.25	.10	.28
12	—	.32	.38	.21	.30

Uric acid content of saliva.—Daily saliva samples from each of twelve subjects were collected for four five-day periods. Equal volumes of each sample were pooled for a five-day period. For analysis of uric acid in saliva, chromatograms were prepared using 10, 15, 25, and 55 microliters of saliva alternating with standards containing uric acid added to five microliters of saliva. If uric acid could not be detected in 55 microliters of saliva, no attempt was made to determine it by using larger volumes.

Table III shows the results of this study. Less than half of the subjects consistently secreted uric acid in excess of the minimum detectable quantity of .0025 mg. per ml. There is at least a 32-fold difference between the six subjects showing no detectable uric acid secretion and subjects 5 and 6 who secreted an average of 0.08 mg. per ml. There is a 10-fold difference between subjects 5 and 6, and subject 7, whose average was 0.008 mg. per ml. Admitting a possible error of 50% in determinations at these low concentrations, the differences noted are still highly significant.

Uric acid determinations have been made on single and duplicate saliva samples from a number of individuals in addition to those listed in Table III. In all, saliva samples from 66 individuals have been studied. Of these, 25 secreted less than 0.0025 mg. of uric acid per ml.; 4 secreted in the range from 0.0025 to 0.01 mg. per ml.; 29 secreted in the range from 0.01 to 0.05 mg. per ml.; and 8 secreted in the range from 0.05 to 0.13 mg. per ml.

TABLE III

Secretion of Uric Acid in Saliva

(mg./ml.)

Subject	*First Week*	*Second Week*	*Third Week*	*Fourth Week*	*Average*
1	*	*	*	*	*
2	*	*	*	*	*
3	*	*	*	*	*
4	*	*	*	*	*
5	0.037	0.042	0.09	0.15	0.08
6	0.050	0.052	0.15	0.068	0.08
7	0.010	*	0.0025	0.020	0.008
8	*	*	*	*	*
9	0.0075	*	*	*	*
10	0.037	0.055	0.063	0.050	0.05
11	*	*	*	*	*
12	—	0.05	0.022	0.013	0.03

* Less than 0.0025 mg./ml.

Summary.—A method is described for the quantitative determination of uric acid in urine by a paper chromatographic method, employing 2,6-lutidine as a solvent and ammoniacal silver nitrate solution as the color developing reagent. The average error in recovery experiments was 17%. The method has been applied to the determination of uric acid in weekly pooled urine and saliva samples from 12 subjects. The variation in the weekly urinary uric acid excretions of a given individual was greater than the variation of the averages of the different subjects. Variations in diet are doubtless important in this connection. Highly significant variations between individuals were noted in the salivary secretion of uric acid.

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IX. The Quantitative Estimation of Urea by Paper Chromatographic Methods with Application to Human Urine

by

HELEN KIRBY BERRY

As the principal nitrogenous end-product of protein metabolism in mammals, urea is of central importance in any thorough study of urine composition. The migration and identification of urea on paper chromatograms of urine have been previously described by Dent (1).

Experimental

Phenol-hypochlorite color reagent.—On urine chromatograms prepared using phenol as a solvent, a bright green spot with Rf value of .80 appeared after spraying with "Clorox" (commercial grade contains 5.25% sodium hypochlorite). This spot was in the position occupied by urea as developed with mercuric nitrate-sodium hydroxide, the color developing reagent used by Dent (1). Identical Rf values were obtained with the two color reagents using either pure urea solutions or urine, and regardless of the solvent employed in developing the chromatogram. To confirm the identity of the substance reacting with sodium hypochlorite, 10 ml. of urine to which 0.35 g. of urea had been added was incubated overnight with urease and then chromatographed. No urea could be detected on the chromatograms of the treated urine, using either color reagent.

In addition to the green spot at Rf .80, all chromatograms prepared using phenol as solvent and developed with sodium hypochlorite showed a blue spot at Rf .28, which appeared to correspond to a strong acid anion, chloride or sulfate. The qualitative appearance of the urine chromatograms could be duplicated by chromatographing synthetic mixtures of urea and ammonium chloride.

The phenol-hypochlorite reagent is a color reagent of rather wide application. It has been used in a specific test for glycine (2), and for the detection of small concentrations of ammonium ion (3). A number of compounds occurring in urine, which might possibly interfere with the determination of urea, were tested for reaction with phenol and sodium hypochlorite. Those which developed a

color included: allantoin, arginine, uric acid, creatinine, glycine, taurine, *p*-aminobenzoic acid, tryptophan, histamine, pantothenic acid, lactic acid, tartaric acid, HCl, NaCl, NH_4Cl, H_2SO_4, and $(NH_4)_2SO_4$. The color developed and the Rf values for these substances are recorded elsewhere in this bulletin (4). None of the substances with Rf values similar to urea are present in urine in sufficient concentration to cause substantial interference.

The color forming reaction between urea and sodium hypochlorite does not occur in the absence of phenol. The color and intensity of the spot is markedly affected by the amount of phenol present on the sheet when sprayed with sodium hypochlorite, too much phenol resulting in very faint spots, and too little resulting in spots which fade rapidly to a yellow-green. With too little phenol present the background of the whole sheet turns brown almost immediately. When phenol is used as the solvent in urea determinations, it is therefore important to control carefully the drying of the sheet prior to color development. Best results have been obtained by drying the sheet for eight minutes at 90° C. When properly developed the green color of the urea spot remains stable for long periods, but the background may slowly turn brown due to the action of sodium hypochlorite on the filter paper.

Selection of solvent.—The following solvent mixtures were investigated: (a) 80 ml. of *n*-butanol, 20 ml. of ethanol, and 20 ml. of water; (b) 65 ml. of 2,6-lutidine and 35 ml. of water; and (c) 100 g. of phenol and 20 ml. of 10% sodium citrate solution (5). Chromatograms run in the first two solvents were sprayed with a solution of 5% phenol in 95% ethanol and allowed to dry before spraying with sodium hypochlorite. Using 2,6-lutidine, urea migrates to an Rf value of .43, but the background turns blue very soon after spraying with sodium hypochlorite. With the butanol-ethanol solvent, urea migrates to an Rf value of .50, and the acid anion spot appears at Rf .24. In the phenol-citrate solvent urea appears at Rf .80 and the acid anion moves only slightly to Rf .15. The phenol-citrate solvent was chosen for general use because the wide spread in Rf values between the two substances in urine which develop color makes possible their rapid separation using short ascents. The use of phenol as solvent also eliminates the phenol spraying step which would be required if any other solvent were employed.

Chromatographic assay procedure.—The general techniques employed in preparing and developing the paper chromatograms

have been described elsewhere (5, 6). Because of the total lack of interfering substances of similar Rf value it is convenient to use short paper sheets; an ascent of 1½ to 2 inches by the solvent front is sufficient to separate clearly the urea spot from the acid anion spot. Sheets 13 x 2½ inches were employed, providing sixteen positions for application of sample and standards at ¾ inch intervals, ½ inch from the bottom of the sheet. Only 20 minutes is required for the solvent to ascend to within ¼ inch of the top of the sheet.

The spot comparison method, described in detail elsewhere in this bulletin (5), was employed for quantitative estimation. Spots developed from five microliters of 1:2 and 1:4 dilutions of urine were compared with adjacent spots developed from five microliters of 1:4 and 1:8 dilutions of urine plus added urea. The eight standard spots on a sheet were made up to correspond to 10, 15, 20, 25, 30, 40, 50, and 60 mg. urea per ml. urine. Having determined the approximate urea content of a given urine sample on such a chromatogram, the concentration can be more accurately determined on a second sheet on which the standard spots are present at two mg. per ml. intervals.

The necessity for the first "rough" chromatogram is eliminated if the urea content of the sample can be estimated by other means. A sufficiently accurate estimate for this purpose can usually be made, based on the specific gravity of the urine. Table I shows the range of urea concentrations usually encountered for a given specific gravity range.

TABLE I

Specific Gravity and Urea Content of Urine

Specific Gravity Range	*Probable Range of Urea Conc. (mg./ml.)*
Below 1.006	4-10
1.006-1.012	8-20
1.012-1.032	16-40
Above 1.032	32-80

It is important that the standard spots be developed in the presence of urine. Creatinine present in the urine, although it does not form a colored spot with phenol-hypochlorite, does affect the Rf value of the urea spot slightly, and also causes a somewhat more diffuse spot.

Results

Recovery experiments.—The results of recovery experiments on three different urine samples are shown in Table II. For each

determination a preliminary chromatogram was run to determine the approximate urea content, and then a second chromatogram with smaller intervals between standards was used to obtain the recorded value. The recoveries varied from 87-102% with an average value of 96%.

TABLE II

Recovery from Urine of Added Urea

	Urea Added (mg./ml.)	*Urea Found (mg./ml.)*	*Urea Recovered (mg./ml.)*	*Percent Recovery*
Sample 1	0.0	10	0	
	16.7	26	16	96
	26.7	36	26	97
Sample 2	0.0	19	0	
	13.3	32	13	98
	33.4	48	29	87
Sample 3	0.0	9	0	
	12.1	20.5	11.5	95
	28.0	37.5	28.5	102

Urea content of male urines.—As a further test of the method, morning urine samples were collected from twelve men for five days a week over a four-week period. The samples from each subject for each week were pooled on the basis of a constant creatinine content. The results of this study are shown in Table III. Values for the urea content are recorded as mg. of urea per mg. of creatinine, in order that the subjects may be compared on a basis more nearly independent of urine volume. Creatinine was determined by a colorimetric method (9). The week to week ratios, urea:creatinine, sometimes vary for a given individual by a factor of two. It will be noted that the individual and average values for subjects No. 1 and No. 6 are much lower (average a little over

TABLE III

Urinary Urea/Creatinine Ratios for Twelve Males

Subject	*First Week*	*Second Week*	*Third Week*	*Fourth Week*	*Average*
1	7	9	12	13	10
2	14	17	16	9	14
3	19	20	15	8	16
4	13	15	—	13	14
5	13	14	16	23	16
6	8	10	12	9	10
7	21	13	14	11	15
8	13	15	21	12	15
9	17	18	19	19	18
10	14	12	21	13	15
11	17	17	18	24	19
12	—	11	12	10	11

50%) than those of subjects No. 9 and No. 11. This seems to indicate, since the diets were self chosen, that subjects No. 9 and No. 11 consistently tend to eat high protein diets compared with subjects No. 1 and No. 6.

Summary.—A method is described for the quantitative determination of urea in urine by a paper chromatographic method, employing phenol as a solvent and phenol-hypochlorite as the color developing reagent. Recoveries of urea added to urine average 96%. The method has been applied to the determination of urea in weekly pooled urine samples from 12 males.

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X. A Micro Determination of Sodium Using Paper Chromatography

by

HARRY ELDON SUTTON

In working with biological fluids, it is frequently desirable to estimate the sodium concentration when only a small amount of material is available. To accomplish this, the following test has been developed which involves only a few micrograms of sodium and requires only a small amount of inexpensive equipment. The analysis employs paper chromatography and is based on the highly specific and sensitive reaction of sodium with zinc uranyl acetate to form a fluorescent compound. The sodium is quantitated by visual comparison with a standard on the same chromatogram.

Experimental Procedure

Choice of solvents.—In testing various solvent mixtures for the resolution of sodium, it was found that the sodium tends to migrate to the positions occupied by the various anions present. With neutral solvents this makes comparison with a standard composed of one substance impossible because of the multiple spots. To overcome this, strongly acidic solvents were tried so that the sodium would migrate almost exclusively to one position with the one anion. The solvent which was decided upon consists of 80 parts of 95% ethanol to 20 parts of 0.1 *N* hydrochloric acid. In this solvent, the sodium migrates to an Rf value of .68. The solvent mixture can be used repeatedly.

Preparation of chromatograms.—Since the sodium separates from the interfering substances rather rapidly, it is not necessary to allow the solvent to rise very high. Sheets about 12 cm. high and 26 cm. wide are used with the spots placed 2.5 cm. from the bottom of the chromatogram and 2 cm. apart. Six spots each of standard and unknown are placed on the chromatogram in alternate positions, the standard occupying the first spot on the right and decreasing in concentration to the left and the unknown occupying the first spot on the left and decreasing in concentration toward the right. For the tests reported here, urine in 1:8 dilution was used as the unknown. The quantities used were 4, 5, 6, 7, 8, and 9 microliters, no more than five microliters being

applied at a time. For the standard, similar volumes of a sodium chloride solution (1.00 mg./ml.) were used. The solvent is allowed to rise to about one cm. from the top of the sheet (60-90 minutes). In selecting the standard the following salts were tested in concentrations containing equivalent amounts of sodium: NaCl, Na_2SO_4,$NaNO_3$, sodium acetate, $NaHCO_3$, sodium hydrogen tartrate. There was no visible difference in the ability of the salts to form the fluorescent compound.

Color development of the chromatograms.—After the chromatograms are dried, they are developed by either of two methods with a solution of zinc uranyl acetate prepared according to directions given on page 32. Care must be taken in preparing this reagent according to directions, otherwise the reagent may not be sufficiently sensitive to the amounts of sodium to be detected. The preferred method of development is to spray the chromatogram lightly with the reagent. The spray should be very fine, and it is only necessary to develop the area to which the sodium is known to migrate (detectable by a thin yellow line due to a second acid front). The reagent should be sprayed only if adequate facilities are available to prevent contact of the zinc uranyl acetate with the skin. An alternate method of development is to dip the chromatogram into the reagent. This procedure can give equally as good results as spraying, but greater precautions must be taken to prevent contamination with sodium from the hands or other objects. The chromatogram should not be allowed to touch the dipping tray as this causes the spots to spread as well as causing contamination. Rubber gloves should be worn if the chromatograms are to be manipulated by hand.

Marking the chromatogram.—After the chromatograms have been developed they are observed under ultraviolet light, the sodium appearing as bright blue fluorescent spots. If the chromatograms were dipped, they should be observed immediately to avoid streaking; if they were sprayed, they are more easily observed in the dry state. The sodium in the unknown is quantitated by selecting the two adjacent spots which appear to be equal in intensity and equating the amount of sodium in them. Thus if a spot of the urine sample is equal in intensity to seven micrograms of standard sodium chloride, then from the known volume and dilution of the urine, the original concentration of sodium in the urine can be calculated. The spreading of the sodium during resolution is a function of the concentration, and the area of the spot is considered along with the intensity of fluorescence in

matching spots. It is sometimes possible to estimate amounts by interpolation.

Expression of results.—Table I has been used to convert the results of the chromatograms into concentration of sodium in the unknown. The figures given as concentration of sodium should be multiplied by the appropriate concentration or dilution factor and rounded off to the proper number of significant figures to obtain the value of sodium in the original unknown. Assuming no error in matching adjacent spots, the maximum error in the extremes of the table would be 11% with the accuracy increasing toward the center. In practice, if sufficient precautions are taken, it is possible to stay within this maximum theoretical error.

TABLE I

Table for Converting Matched Spots on Chromatograms into Concentration of Sodium.

Volume Unknown (μl)	*Volume Standard (μl)*	*Conc. Na in Unknown (mg./ml.)*
9	4	0.176
8	4	0.198
8	5	0.246
7	5	0.282
7	6	0.341
6	6	0.397
6	7	0.464
5	7	0.556
5	8	0.635
4	8	0.794
4	9	0.893

Results

To determine the accuracy of the method as applied to urine, several samples were analyzed by one experimenter using this technique, and the values recorded. These samples were then diluted 1:2 and sodium chloride was added by a second experimenter in amounts unknown to the first experimenter but sufficient to restore the concentration to the range normally found in urine. These altered samples were then analyzed as "unknowns," single determinations being made. The results are recorded in Table II.

The average error for these nine determinations is 9.4%. The first five samples were sprayed and the last four dipped, but there is no significant difference in accuracy. Since the original value of the samples was also determined by this method, part of the error may undoubtedly be attributed to that source.

TABLE II

Comparison of Experimentally Determined Sodium Concentrations with Known Values.

Sample	*Sodium found in original samples (mg./ml.)*	*Sodium in diluted original plus added amount (mg./ml.)*	*Sodium as determined (mg./ml.)*	*Per Cent recovery*	*Per Cent error*
1	4.1	3.2	3.5	109	9
2	4.8	2.7	3.2	118	18
3	2.3	1.7	1.8	106	6
4	3.2	2.6	2.7	104	4
5	3.7	2.6	2.3	88	12
6	4.1	2.7	2.9	107	7
7	4.8	4.4	3.7	84	16
8	3.2	3.8	3.7	97	3
9	3.7	4.1	3.7	90	10

Discussion

The methods in use at present for the determination of sodium generally require expensive equipment or involve considerable time. Perhaps the chief advantage of the method presented here is that it requires neither. The results of a given determination can be obtained within two hours, and if many determinations are to be done, one person can average five to ten determinations an hour. The materials required are very simple and easy to obtain. The accuracy of the test compares rather favorably with that of other techniques, especially when the range of concentrations is known in advance.

Another advantage of this technique is the small amount of unknown material required. For most samples of human urine, only 39 microliters of a 1:8 dilution are actually used. This applicability to very small amounts becomes particularly advantageous, for example, when working with small animals. In addition to use in the analysis of urine, the method should also be adaptable to such materials as saliva, blood, tissue extracts, etc.

XI. Quantitative Study of Ketosteroids by Paper Chromatographic Methods with Applications to Human Urine

by

ERIC BLOCH, ERNEST BEERSTECHER, JR., AND ROY C. THOMPSON

Zaffaroni, Burton, and Keutmann have described a method for the qualitative separation of ketosteroids by paper partition chromatography (1). The critical feature of their method lies in the fact that prior to being chromatographed the ketosteroids are converted to their hydrazone derivatives through the use of Girard's Reagent T (trimethylaminoacetohydrazine) (2). Water solubility is enhanced by this treatment to a varying degree depending on the number of carbonyl groups present in the original molecule, and consequently the spread of Rf values for the various ketosteroids is increased. The problem of locating the ketosteroid spots on the developed chromatogram is also simplified by the presence of the reactive quarternary nitrogen groups which produce colored compounds by reaction with suitable alkaloid reagents.

In the present study, Zaffaroni's method has been further investigated and adapted to the quantitative estimation of ketosteroids.

Experimental

The reagents used and their methods of preparation were essentially those described by Zaffaroni, *et al.*, (1). Hydrazones were formed by Zaffaroni's alternate "Method B" in which the ketosteroid, Girard's Reagent T, and acidified methanol were incubated at 40° C. for two hours or more. Zaffaroni employed water-saturated *n*-butanol and a 1:1:1 mixture of *tert*-butanol, *n*-butanol, and water as developing solvents. In the present investigation a large number of solvent mixtures were studied over a wide range of composition ratios. Best results were obtained using a mixture containing 84 parts *n*-butanol and 16 parts water (which is essentially a water-saturated solution). A *tert*-butanol-*n*-butanol-water mixture in a volume ratio of 1:2:1 also proved satisfactory. The techniques employed in preparing and developing the paper chromatograms have been described elsewhere (3, 4). Color was de-

veloped with K_2PtI_6 as described by Zaffaroni, *et al.* (1). A general description of the quantitative procedures employed will be found elsewhere in this bulletin (4). Quantitative inferences were drawn from the area of the developed spots. Since the color developed with the K_2PtI_6 solution fades rather rapidly on drying, the outline of the spot was marked with a pencil while the sheets were still damp.

Urine samples for determination of ketosteroids were obtained from 14 subjects, seven male and seven female. One of the subjects (No. 6) was in the eighth month of pregnancy. Approximately 800 ml. of urine were collected from each subject. Samples collected after periods of sleep were not included. A modified form of the Pincus method of hydrolysis and extraction (5) was followed in the separation of the ketosteroid fractions from the urine samples. To 750 ml. of urine, 110 ml. of concentrated hydrochloric acid were added and the mixture was refluxed for seven to nine minutes. The solution was then quickly cooled to room temperature under running water. The hydrolyzed urine was continuously extracted with 220 ml. of diethyl ether for six to seven hours, a modified form of the Hershberg-Wolfe cyclic extractor being used (6). The ether extract was washed successively with 50 ml. of distilled water, twice with 25 ml. of a saturated sodium carbonate solution, four times with 2 *N* sodium hydroxide and three times with 30 ml. of distilled water. The resulting ether extract was evaporated to dryness and the residue dissolved in five milliliters of 10% methanolic-acetic acid. For conversion of the ketosteroids to hydrazones, 20–40 mg. of Girard's Reagent T were added and the solution was incubated at 40°C. for two hours or longer.

Although much pigmented material was removed during the washing of the ether extract, the methanolic-acetic acid ketosteroid hydrazone solution was still highly colored. Most of this color was removed by filtering the solution through Darco charcoal. Any adsorbed hydrazones were eluted by repeated washings with 0.5 ml. of 10% methanolic-acetic acid. For three urine samples, the charcoal treatment resulted in fewer spots on the developed chromatogram. For six samples the number of spots was found to increase after charcoal treatment. All of the data on urine presented in this paper were obtained on charcoal treated solutions. Fifty microliters of the ketosteroid hydrazone solution derived from urine were chromatographed in every case.

Results

The Rf values obtained for a number of pure ketosteroid hydrazones using 84:16 *n*-butanol-water, and 1:2:1 *tert*-butanol-*n*-butanol-water as resolving solvents are listed in Table I. Also included for comparison are the Rf values reported by Zaffaroni, *et al.* (1) using water-saturated *n*-butanol and 1:1:1 *tert*-butanol-*n*-butanol-water. It may be noted that while the 84:16 *n*-butanol-water mixture is essentially the same as Zaffaroni's water-saturated *n*-butanol solvent, there is an appreciable difference in the Rf values obtained. We are unable to give a reason for these variations, but presumably they are due to some difference in the conditions under which the chromatograms were prepared. Of primary interest from the analytical viewpoint is the wider range of Rf values obtained in the present investigation as compared with the results of Zaffaroni. The effect of structure on Rf values pointed out by Zaffaroni is even more clearly shown in the results of this study. The predominant factor contributing to greater water solubility and consequently lower Rf values, is the number of Reagent T groups bound per molecule. Of lesser importance (but clearly of significance as indicated by the range of Rf values from .69 for $\triangle^1$–cholestanone to .52 for estrone) is the number of other polar groups in the molecule and the number of carbon atoms. Confirming Zaffaroni's observation, allopregnane–3,20–dione behaved in a manner comparable to the monoketonic compounds. On the basis of observations on other similar compounds he has suggested that 3–keto groups, without conjugated unsaturation, do not form with Girard's Reagent T hydrazones sufficiently stable to escape hydrolysis during development of the chromatogram.

In the present investigation pregnane–3,20–dione gave no detectable spot when resolved with 84:16 *n*-butanol-water, and only a faint spot of doubtful significance appeared when developed with 1:2:1 *tert*-butanol-*n*-butanol-water. This behavior is contrary to the findings of Zaffaroni, and there would seem to be no apparent reason why this compound should differ chromatographically from its allo-isomer. The appearance of two spots for either progesterone or desoxycorticosterone—one at an Rf value indicative of disubstituted ketosteroids and the other at that of monosubstituted products—may indicate either an insufficient amount of Reagent T present to yield these two diketosteroids in their disubstitued form only, or impurities in the crystalline sample. In the case of the desoxycorticosterone hydrazone, a spot for uncombined Reagent

TABLE I

Rf Values of Ketosteroid-Reagent T Hydrazones

*Ketosteroids**	*Structural Characteristics*					*Rf Values*			
	Carbonyl Groups					*This Investigation*		*Zaffaroni*	
	Reagent T Bound	*Free*	*Hydroxyls*	*Double bonds*	*Carbon atoms*	*84:16 n-butanol water*	*1:2:1 t-butanol n-butanol water*	*"water satd." n-butanol*	*1:1:1 t-butanol n-butanol water*
△[1]-cholestenone	1	0	0	1	27	.69	—	—	—
Dehydroisoandrosterone-acetate	1	0	1	1	21	.63	.84	.49	—
Pregnane-3,20-dione	1	1	0	0	21	—	.91(?)	.48	.70
Allopregnane-3,20-dione	1	1	0	0	21	.61	.86	.48	—
Isoandrosterone	1	0	1	0	19	.58	.83	.49	—
Testosterone	1	0	1	1	19	.56	.81	.49	.69
Estrone	1	0	1	3	18	.52	.92	.47	—
Progesterone	2	0	0	1	21	.19 (.59)	.45 (.84)	.15	.28
Desoxycorticosterone	2	0	1	1	21	.17 (.66)	—	—	—

*Ketosteroids were purchased from Bios Laboratories, with the exception of dehydroisoandrosterone-acetate and △[1]-cholestenone, which were supplied through the courtesy of Schering Corp. and Eli Lilly and Co., respectively.

T (color developed with Tollen's reagent) was noted in the case of one preparation.

In Table II are summarized the results of experiments designed to establish a relationship between the quantity of ketosteroid hydrazone applied and the area of the resultant developed spot. The solvent was 84:16 *n*-butanol-water. The areas recorded are the average of four determinations. Where a single value differed from the mean by more than four times the average deviation of the three remaining values, it was eliminated. The area measurements are quite reproducible except at the five microgram level. The marked effect of temperature on spot area is shown by the two estrone and dehydroisoandrosterone–acetate experiments conducted at 15° and 25° C. Since the temperature in these experiments was controlled only within limits of perhaps ±2° C., the observations should be interpreted accordingly. They indicate the importance of careful control of temperature, or the incorporation of standards on the same chromatograph sheet with unknown samples, if the areas measured are to be of quantitative significance.

TABLE II

Relationship Between Amount of Ketosteroid Hydrazone Chromatographed and Area of the Resulting Developed Spot

Steroid hydrazone	*Micrograms of ketosteroid applied*						*Approx Temp.*
	5	*10*	*15*	*20*	*25*	*30*	
Testosterone							
area (cm^2)	1.08	1.98	3.63	3.60	4.15	5.93	15°C.
avg. dev. (%)	13	9	6	2	1	8	
Rf	.61	.60	.60	.60	.59	.58	
Progesterone							
area (cm^2)	0.54	1.41	1.91	1.99	2.48	2.58	15°C.
avg. dev. (%)	11	12	8	7	7	12	
Rf	.21	.24	.25	.21	.21	.21	
Isoandrosterone							
area (cm^2)	1.76	3.80	5.13	5.43	6.78	7.47	25°C.
avg. dev. (%)	22	4	2	3	4	3	
Rf	.72	.72	.70	.68	.70	.70	
Estrone							
area (cm^2)	—	2.40	3.81	4.32	4.94	5.41	15°C.
avg. dev. (%)	—	7	2	5	—	2	
Rf	.71	.69	.64	.65	.68	.69	
Estrone							
area (cm^2)	1.78	5.98	8.28	9.02	8.95	10.58	25°C
avg. dev. (%)	2	—	3	6	2	5	
Dehydroisoandrosterone-acetate							
area (cm^2)	0.64	2.58	4.04	5.00	6.15	7.46	15°C.
avg. dev. (%)	22	3	3	2	2	1	
Rf	.66	.69	.65	.66	.67	.67	
Dehydroisoandrosterone-acetate							
area (cm^2)	2.68	5.49	7.17	8.37	11.58	12.39	25°C.
avg. dev. (%)	25	7	5	7	—	2	

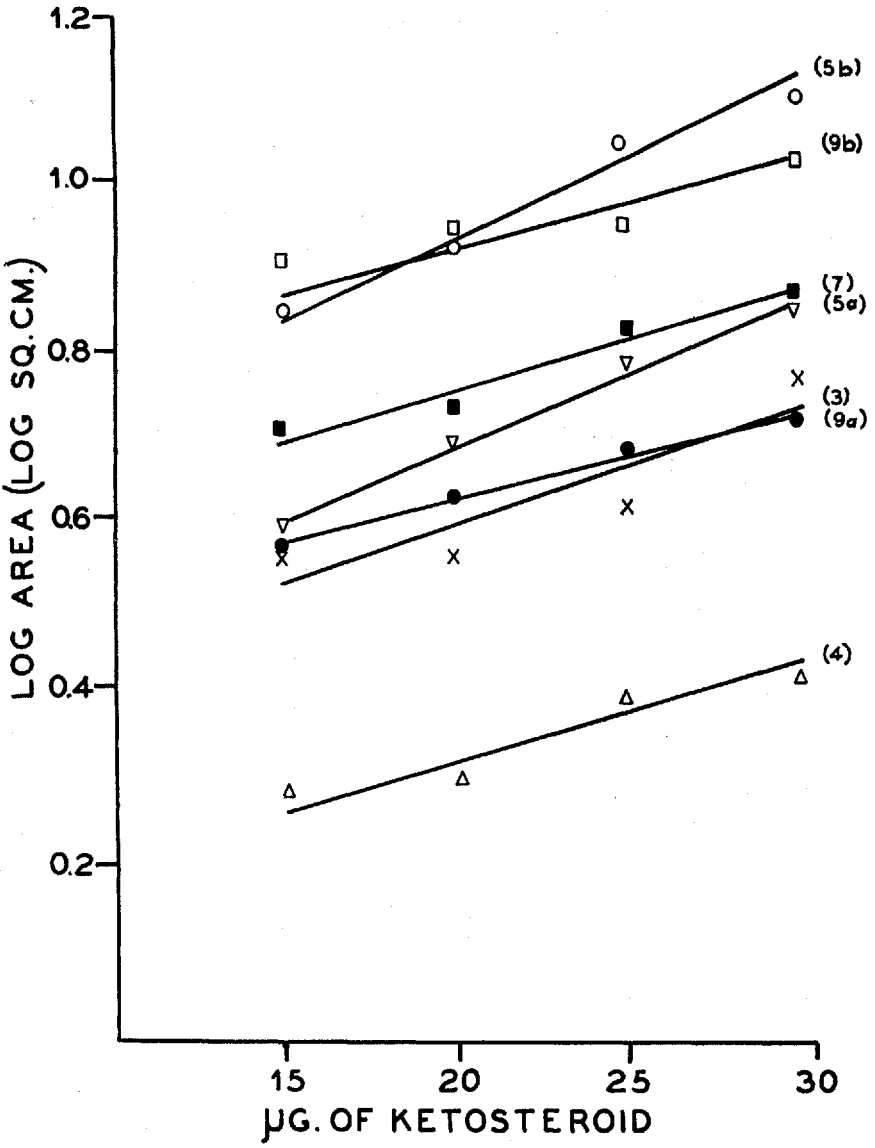

FIGURE 1

Log Graph of the Area of Spots Produced by Varying Concentrations of Ketosteroids. *Solvent:* 84:16 n-butanol-water mixture.

5b = dehydroisoandrosterone at 25° C.
9b = estrone at 25° C.
7 = isoandrosterone at 25° C.
5a = dehydroisoandrosterone at 15° C.
3 = testosterone at 15° C.
9a = estrone at 15° C.
4 = progesterone at 15° C.

Also included in Table II are the measured Rf values for the different ketosteroid hydrazones at different concentrations. No significant change in Rf values with change in concentration is observed.

In Figure 1, the logarithms of the areas recorded in Table II are plotted against micrograms of ketosteroid applied. An approximately linear relationship is obtained except in the lowest concentration range.

Results on Urine Samples

Figure 2 reproduces the patterns obtained by chromatographing 50 μl. portions of four of the urine samples. The shaded spots were purple in color, the degree of shading indicating the intensity of the color. The unshaded spots were gray-black in color. The purple spot (or spots) of high Rf value is in a position to be expected of ketosteroids with one Girard's Reagent T group bound. The purple spots of low Rf value are in a position to be expected of ketosteroids with two or more Reagent T groups bound. The purple ring at Rf. value .00 was not observed in the chromatography of crystalline ketosteroids. The gray spot of lowest Rf value, labeled G.R.T. in Figure 2, is due to uncombined Reagent T. The gray spots of intermediate Rf value are of unknown nature. They might be due to ether soluble pigments, but they were not affected by charcoal treatment, nor does their position correspond to that of any of the several fluorescent spots which were visible under ultra-violet light.

The apparent multiple character of the purple spot of high Rf value led to obvious difficulties in interpretation and in the measurement of areas. The line of demarkation between the deep purple spot and the lighter purple areas bordering it on either side (in most of the chromatograms) was quite sharp. The area of the darker spot could therefore be determined quite accurately. Areas of the light purple regions bordering the central spot were determined separately. Despite these difficulties obvious similarities are apparent in the chromatograms from different urine samples, as are also certain dissimilarities.

In Table III are recorded the Rf values and areas of the spots obtained from the 14 urine samples. Recorded values are the averages of duplicate runs. There was excellent agreement between Rf values in duplicate runs, the difference never exceeding .03. Agreement was not so good in the area measurements. Crystalline ketosteroids chromatographed with the urine samples gave the

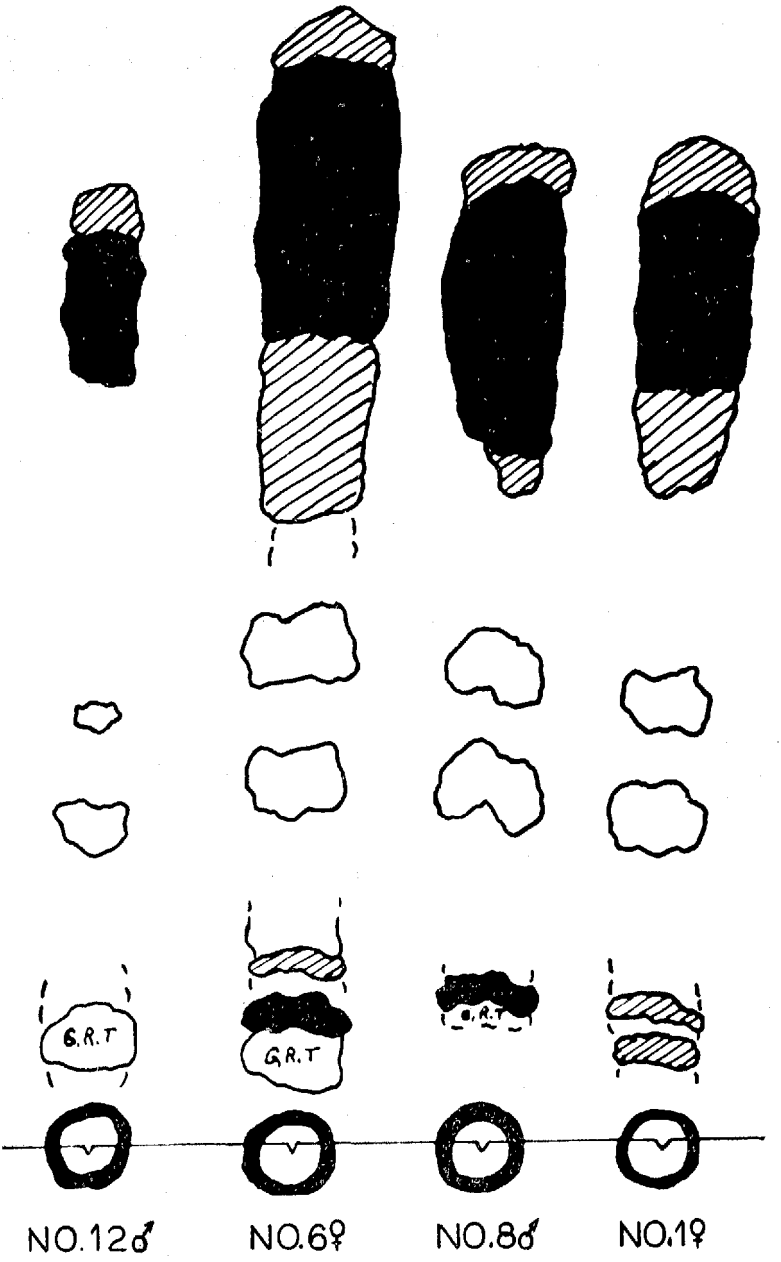

FIGURE 2

Reproductions of the Chromatographic Patterns of Urine from Four Individuals. *Solvent:* 84:16, n-butanol-water solution; developed with K_2PtI_6 solution. *Shaded spots:* spots produced by steroid substances, shading indicating intensity of color; *Colorless spots:* probably ketosteroid in origin but not positively identified.

following Rf values: dehydroisoandrosterone-acetate, .58; testosterone, .55; isoandrosterone, .54; and estrone, .46. The general similarity of all urine samples is clearly indicated in Table III. The outstanding differences observed between samples are (a) the clear cut sex dependence in the case of "spot 7" and (b) the unique pattern of the pregnancy urine (subject No. 6.).

The spots due to ketosteroid hydrazones of low Rf value (spots 6 and 7 in Table III) cannot be identified with any pure ketosteroid studied in this laboratory by paper chromatographic methods. The responsible substances must be capable of binding two or more Girard's Reagent T groups if one is to account for the low Rf values. Of the diketosteroids which have been isolated from human urine (7, 8), apparently none is capable of combining with more than one Reagent T group. Zaffaroni, *et al.* (1), have shown that 3–keto groups without conjugated unsaturation do not form stable hydrazones, and Lieberman, *et al.* (8), have recently presented evidence to indicate that the same situation exists in the case of 11–keto groups. Progesterone and desoxycorticosterone are known to form dihydrazones; the Rf values of these pure dihydrazones, however, are higher than those of spots 6 and 7 (Table I). Furthermore, steroids of this type are not thought to be present in normal human urine. Regardless of the identification of these spots, the absolute sex dependence of spot 7 is of great interest.

There is some question as to whether spot 6 from female urine samples should be considered identical with spot 6 from male samples, the Rf values being consistently higher in the case of the male samples (excluding from consideration the pregnancy urine). The difference is very slight, however, and they may be the same.

Spots 1, 2, and 3 (Table III) are undoubtedly due to 17-ketosteroids which constitute the great bulk of ketosteroids excreted in urine, i.e., androsterone, isoandrosterone, dehydroisoandrosterone, etiocholane–3(*a*)–ol–17–one, etc. Whether spots 1, 2, and 3 represent overlapping spots due to individual ketosteroids, or a single diffuse spot due to a mixture of substances, is uncertain; and this uncertainty makes any precise quantitative interpretation impossible. Considering only the area of the central deep purple spot (spot 2) and using a standard response curve for isoandrosterone, values for 17–ketosteroid content in mgs. per liter of urine have been calculated and are given in Table IV. One would expect these values to be low, since the whole area of the spot (or spots) has been employed.

TABLE III

Rf Values and Areas of Spots from Chromatographed Urine Extracts

Female Urine Samples	*Spot 1 (purple)*		*Spot 2 (purple)*		*Spot 3 (purple)*		*Spot 4 (gray)*		*Spot 5 (gray)*		*Spot 6 (purple)*		*Spot 7 (purple)*	
	Rf	*Area*	*Rf*	*Area*	*Rf*	*Area*	*Rf*	*Area*	*Rf*	*Area*	*Rf*	*Area*	*Rf*	*Area*
1	.61	1.1	.53	4.7	.43	1.4	.27	0.9	.21	1.3	.09	0.29	.063	0.46
2	.60	1.1	.53	2.8	—	—	.27	x	.20	0.5	.085	0.39	.066	0.45
3	—	—	.56	3.2	—	—	.27	0.4	.21	0.5	.089	0.25	.075	0.29
4	.65	1.2	.56	5.7	.46	1.6	.27	0.5	.20	0.8	.085	0.36	.065	0.44
5	.61	0.9	.52	3.9	.43	0.8	.27	x	.20	0.5	.081	0.37	.065	0.32
6*	.71	0.7	.60	8.2	.45	4.4	.31	1.5	.22	1.4	.115	0.30	.087	0.70
7	.60	1.0	.53	3.1	.47	0.3	—	—	.20	0.2	.087	0.25	.067	0.41
Male														
8	.61	1.1	.54	4.5	.45	1.6	.30	1.5	.22	1.5	.094	0.54	—	—
9	.62	1.0	.53	6.0	.41	0.7	—	—	.21	0.4	.096	0.47	—	—
10	.60	1.1	.53	3.6	—	—	—	—	—	—	.091	0.34	—	—
11	.62	1.1	.53	4.5	.43	0.6	.29	0.5	.22	1.2	.096	0.42	—	—
12	.59	1.4	.53	2.5	.47	x	.27	x	.20	0.6	—	—	—	—
13	.62	1.2	.51	4.5	.41	0.7	.28	0.5	.22	1.1	.093	0.30	—	—
14	.63	0.7	.55	5.2	.46	0.9	.29	0.5	.22	0.5	.110	0.44	—	—

*pregnancy urine.
x spot too small for precise quantitative measurement.

Not included in the above table are gray spots with an Rf value of .38-.39 observed in three urine samples, nor the spots due to unreacted Reagent T with Rf values of .04-07.

The comparison of these semi-quantitative data with the published results of others is complicated by the method of urine collection employed in this study. The averages of 1.8 mg. of 17-ketosteroids per liter of daytime urine for females, and 2.4 mg. per liter of daytime urine for males, compare with the following

TABLE IV

Quantitative Estimation of 17-Ketosteroids in Urine

	Urine Sample	*17-Ketosteroid (mg/liter)*
Female	1	1.9
	2	1.0
	3	1.2
	4	2.5
	5	1.5
	6*	5.8
	7	1.1
Female Avg. (excluding 6)		1.8
Male	8	1.8
	9	4.2
	10	1.3
	11	1.8
	12	0.8
	13	2.0
	14	2.2
Male Avg		2.4

*pregnancy urine.

values obtained for normal young adults by other workers:

"androgen":
male: 3.8 mg./l. (9), 9.9 mg./day (10), 9.05 mg./day (11)
female; 4.2 mg./l. (10), 6.75 mg./day (11)

androsterone:
male: 1.6 mg./l. (12), 1.2 mg./l. (14), 2.9 mg./day (13)
female: 1.3 mg./l. (13)

eticholane–3 (α)–ol–17–one:
male: 1.4 mg./l. (12)
female: 1.3 mg./l. (12)

dehydroisoandrosterone:
male: 0.2 mg./l. (12), 2.0 mg./day (13)
female: 0.2 mg./l. (12)

It would seem reasonable to conclude that, while the quantitative estimates of the present investigation are no doubt low, they are nevertheless of the correct order of magnitude and sufficiently precise to permit of valid comparison between individual ketosteroid excretion patterns.

The urine sample standing in unique contrast to all others tested is the pregnancy urine (No. 6). The very large "17-ketosteroid spot" is probably due largely to steroids of the pregnane

series which are found in large amounts in pregnancy urine and which would be expected to be chromatographically indistinguishable from the 17–ketosteroids.

Summary.—Zaffaroni's method for separating ketosteroids by paper partition chromatography has been adapted for quantitative estimation of such compounds. The logarithm of the area of the spots obtained on the chromatogram was shown to be linearly related to the amount of ketosteroid chromatographed, for amounts in excess of 15 micrograms. The areas of the spots were markedly affected by temperature, but were quite reproducible under constant environmental conditions for amounts of 10 micrograms or more.

Ketosteroid fractions of the urine of 14 subjects were studied using this method. The chromatograms showed general similarities, but qualitative and quantitative evidences of individuality were apparent. All female urines were characterized by two ketosteroid hydrazone spots of low Rf value, while only one such spot was present on chromatograms from male samples. The single pregnancy urine sample was marked by an increased content of ketosteroid hydrazones migrating with high Rf values. Quantitative estimation of the ketosteroid content of the urines was hampered by diffuseness and/or overlapping of the principal spots, and by uncertainty as to their identification with known ketosteroids.

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XII. The Determination of Ferric Chloride Chromogens in Human Urine by Paper Chromatographic Methods

by

HARRY ELDON SUTTON AND ERNEST BEERSTECHER, JR.

Ferric chloride has long been used as a qualitative reagent for the identification of certain classes of compounds, such as phenols, oximes, enols, etc., and tables of compounds and their corresponding colors with ferric chloride can be found in the literature (1, 2). Ferric chloride would therefore seem to be a useful reagent for the development of color with certain compounds separated on paper chromatograms. The present investigation is an application of this reagent to the qualitative, and, where possible, quantitative determination of certain constituents of human urine on paper chromatograms.

Experimental

The general chromatographic techniques employed have been previously described (3, 4). The solvent employed for the development of the chromatograms was a mixture of four parts *n*-butanol, one part glacial acetic acid, and one part distilled water. Other solvents commonly used in paper chromatography such as phenol, collidine, and pyridine, could not be used since the solvents themselves react with ferric chloride to form colored products.

The paper used was 28 cm. in height. An aliquot of urine containing 40 to 60 micrograms of creatinine was used as the test amount. This volume contained enough of the chromogenic material to react fully and not too much to prevent good resolution.

Chromatograms were allowed to dry in air. Warm air was sometimes passed over the sheets to speed the process, but they were never subjected to the higher temperatures for longer than five minutes. Color was developed by spraying the sheets with a one percent aqueous solution of ferric chloride. This concentration gives maximum color, while keeping the background color at a minimum. With the exception of tartrate, the spots were not clearly visible until the water had been removed from the sheets. Rapid drying in warm air seemed to deepen the colors. As the maximum color intensity was obtained immediately after drying,

the chromatograms were marked as soon as possible after color development.

Quantitative estimates, where possible, were based on the areas of the developed spots. The techniques involved and a general consideration of this procedure will be found elsewhere in this bulletin (3).

Results

Qualitative identification of ferric chloride chromogens in urine. —The spots obtained by spraying urine chromatograms with ferric chloride solution are summarized in Fig. 1. All of these spots did not appear in the urine of every person, nor in all samples from a given person. Most of them, however, appear to be normal daily constituents of urine. The identification of these spots with known compounds involved the paper chromatographic testing of over 125 different compounds known to be, or possibly, present in urine. These included the naturally occurring amino acids, a large number of sugars, organic acids, vitamins and other metabolites, and many inorganic salts. The results of these tests are recorded elsewhere in this bulletin (5). In addition to this study of pure compounds, a "synthetic urine," with a composition falling within the normal range of variation of the major urine constituents (6), was prepared and studied.

While several of the amino acids showed chromogenic activity, only faint spots were obtained even at concentrations much higher than would be expected to occur in urine, and none of the Rf values corresponded to those of similarly colored spots on urine chromatograms. None of the sugars showed color formation at concentrations corresponding to or even higher than those occurring in urine.

Sodium tartrate gives a bright yellow spot at Rf .25 which disappears on drying the sheet. This Rf value and the unique behavior on drying correspond to spot E of Fig. 1, which is therefore ascribed to tartrate. Citric acid corresponds in Rf value and color to spot F of Fig. 1. This spot is usually very faint in urine samples and is difficult to mark accurately.

The sodium salts of lactic acid, acetic acid, and α-ketoglutaric acid give brown spots which correspond very closely to spot G. All sodium salts of weak acids seem to give such a brown spot, Na_2CO_3 and $NaHCO_3$ behaving similarly. Presumably these salts of weak acids react with the acetic acid in the solvent to form sodium acetate which migrates corresponding to an Rf value of about .35, where it reacts, upon spraying with ferric chloride solution, to

precipitate ferric hydroxide. Other ions such as magnesium, potassium, calcium, and cadmium give the same effect as sodium and may contribute to this "alkaline" spot. Spot G may therefore

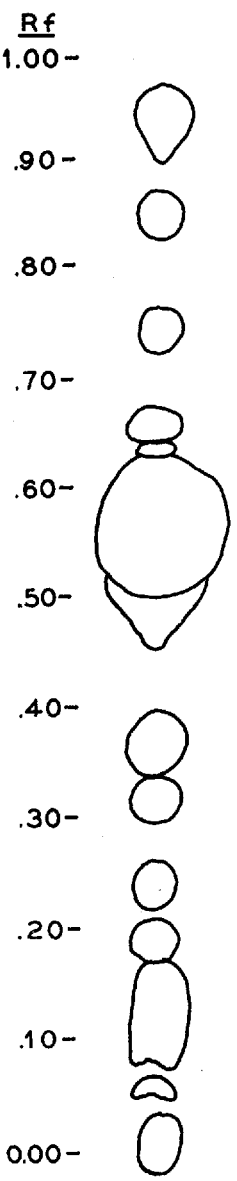

FIGURE 1

Spots Obtained from Urine with Ferric Chloride Chromatograph.

be considered a measure of the weak acids present in the urine. Creatine, which migrates to the same position as spot G and which gives a color similar to the alkaline salts, occurs in such small quantities in normal urine that its contribution to spot G may be ignored.

Of the vitamins tested, pyridoxine and *p*-aminobenzoic acid show the greatest chromogenic activity, one microgram of each being capable of producing a clearly defined spot. These spots correspond roughly to spots found in most urines—pyridoxine to spot K and *p*-aminobenzoic acid to spot M—but the urinary excretion of these substances is so low that it is difficult to draw definite conclusions. These spots found on urine chromatograms are usually too faint to mark accurately and while they may be presumed to be caused by the two vitamins, they cannot be safely used as a measure of excretion of these vitamins.

Urea produces a bleached area of Rf .60. The minimum detectable quantity is about 30 micrograms or the amount present in about one microliter of urine. Spot 1 of Fig. 1 may therefore be assigned to urea. Creatinine, which corresponds to spot H, may best be demonstrated by use of the picric acid reagent (7) which is specific for creatinine and which forms the basis for the standard colorimetric test for creatinine. By the use of this reagent, it can be shown that the creatinine in urine and spot H always occupy the same positions, are always distorted in the same manner by excessive concentrations of urea, and are always of the same relative concentrations on the basis of color intensity. Salicylic acid, which corresponds to spot N, gives an intense purple color with ferric chloride—a fact which is the basis for the clinical test for salicylates, which appear in the urine only after consumption of salicylate drugs (8). The specificity of the ferric chloride color reaction together with the intense fluorescence leave little doubt as to the identity of salicylic acid with spot N.

Inorganic constituents of urine account for three more of the urinary chromogens; sulfate corresponds to spot A, phosphate to spot C, and chloride to spot D. Not all of the different salts of the same acid show the same ability to produce spots. As most of the urine acids are present as sodium and potassium salts, these are of particular importance in establishing the identity of the spots. The identity of the phosphate spot may be best established by spraying the chromatogram with the specific ammonium molybdate reagent (9). The spot produced by such a procedure coincides with spot C. Pure chlorides give no color reaction with ferric

chloride. However, repeated tests with synthetic urines and the use of color developing reagents such as bromcresol green indicate that spot D must be due to chloride, perhaps in combination with some other constituent of urine. The mechanisms of the chloride and sulfate reactions are not clear.

The remaining spots, B, J, and L, have not been identified with any known substance. Spots B and L are very faint and frequently cannot be detected. Spot J is a bright bluish green, usually small and clearly defined, but seldom occurring in quantities sufficient to be detected with ferric chloride. The reactions of this chromogen with other reagents are reported elsewhere in this bulletin (10).

Quantitative estimation of phosphate and urea.—Most of the ferric chloride-developed spots on urine chromatograms are of such a shade of color as to make color intensity comparisons very difficult. This is particularly true in view of the yellow ferric chloride background. Many of the spots are also so ill-defined as to make area measurements impractical. Only in the case of phosphate and urea has it been found possible to devise suitable procedures for quantitative estimation. This is not to say, of course, that gross quantitative information of a relative nature cannot be deduced for many of the other constituents.

For phosphate, a reasonably linear relationship was observed between the amount of phosphate chromatographed and the area of the resulting spot. Comparison of the areas of urinary phosphate spots with the areas of standard phosphate spots to obtain quantitative values of the phosphate concentration gives results which average forty to fifty percent too high when compared with the values obtained on the same samples by standard colorimetric procedures (9). Nevertheless, the chromatographic detection of phosphate coupled with its approximate determination has proved to be useful in studies involving large numbers of urine samples, particularly since from the same chromatogram other valuable information can also be derived.

For the quantitative estimation of urea, the spot comparison technique (3) was employed, spots being matched on the basis of visually estimated area only. Spots developed from five microliters of urine plus varying amounts of urea were compared with adjacent spots from 10 and 15 microliters of urine alone. The results of such determinations on six urine samples are shown in Table I. Values are also given for the urea content of these samples as determined by the standard urease method (10). The average

percent difference between the paper chromatographic determinations and the urease determinations was 14%. The error of the paper chromatographic method could no doubt have been somewhat reduced by measuring the areas of the compared spots with a planimeter and interpolating between standard spots.

TABLE I

Urea Content of Urine
(mg./ml.)

Sample	*Paper Chromatographic Values*	*Urease Values*	*Percent Difference*
1	12	13.5	11
2	18	23.6	24
3	21	19.9	5.5
4	27	23.2	16
5	12	15.3	21
6	7.5	7.9	5.0

Summary.—Spots obtained on paper chromatograms of urine prepared using a *n*-butanol-acetic acid-water solvent and developed for color with ferric chloride have been identified as sulfate, phosphate, chloride, citrate, "weak acid" alkali, creatinine, urea, pyridoxine, *p*-aminobenzoic acid, and salicylate. Three additional spots remain unidentified. Methods are presented for the roughly quantitative estimation of phosphate and the more satisfactory determination of urea.

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XIII. The Effects of Single Vitamin Deficiencies on the Consumption of Alcohol by White Rats

by

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I. *Introduction*

Previous nutritional studies with individual rats (1–3) have shown that their *ad libitum* consumption of 10% ethyl alcohol is highly individualistic and may be profoundly influenced by dietary means. On diets marginal in B vitamins, nearly all of the animals studied consumed large quantities of alcohol, while on the same diets supplemented with a mixture of B vitamins and other growth factors, only a few animals of one strain consumed any alcohol whatsoever. Based on this evidence as well as evidence for the involvement of a genetic mechanism in the appetite for alcohol, the concept of genetotrophic disease was developed (4). As a result of this concept, a treatment has been proposed for alcoholism in humans and subjected to clinical trial (5). In the course of such studies, however, it has not as yet been possible to determine just which growth factors were most specifically involved in controlling the appetite for alcohol, and it has therefore been impossible to study in detail the exact mechanisms involved. A modification of the previous experimental approach was thus expedient in order to obtain further information as to which specific vitamins were involved in the phenomena.

The present study involves the assessment of the role of a number of specific vitamins in relation to appetite for alcohol, as determined with single vitamin deficiency studies with rats, and the evidence presented shows conclusively that the absence of any one of a number of nutritional factors from the diet may cause the development of alcoholic consumption in rats. The results are in line with the genetotrophic concept and also with recent findings relating to the distinctive metabolic patterns in human compulsive drinkers (6).

II. *Experimental*

The experiments were all performed on the two strains of white rats previously described as "O" and "H" (1). The rats were kept

in individual false bottom cages, each equipped with two drinking vessels, one containing 10% by volume of alcohol, and the other water. The positions of the two vessels were interchanged from day to day to rule out the element of habit. The animals were placed on the described deficiency diets, and their weights and consumption of 10% alcohol and of water were measured at two to three day intervals. When the alcohol consumption had arisen to a suitable level, generally about 0.5 ml. absolute alcohol/100 gm. rat/day, the vitamin missing from the diet was administered for a three day period, and the effect on alcohol consumption was noted for a period of two to three weeks before other supplements were tried. When the specifically deficient vitamin failed to abolish the appetite completely, other dietary supplements were tried until final abolition of the appetite was achieved. In some cases, a final cure was achieved only after a period of nearly a year, thus affirmatively answering a question raised earlier (1) of whether a long standing appetite could be cured in older animals.

The diets employed were the standard vitamin deficient diets shown in Table I, all however containing Salt Mixture No. 2 (7) as the mineral supplement. In later experiments, several special diets were prepared as follows:

a. High protein diets were prepared by reversing the usual percentages of casein and carbohydrates; *i.e.*, approximately 68% casein and 18% sucrose.
b. High amino acid diets were prepared by substituting half of the casein (18%) with an enzymatic yeast-protein digest, containing 57% of the total nitrogen as amino nitrogen.
c. Nucleic acid diets were prepared by adding 1.5 gm. of Fleischmann's technical grade nucleic acid to each 100 gm. of the regular deficiency diet or of the other diets as indicated.
d. A typical high protein-nucleic acid-amino acid diet was prepared by combining 60% casein, 17% sucrose, 8% Crisco, 2% cod liver oil, 9% enzymatic yeast-protein digest, 1.5% nucleic acids, 4% salt mixture #2, and the usual vitamin supplement.

At an early stage in the experiments it was found (evidence to be presented in later paragraphs) that the classical deficiency diets in Table I were in many cases deficient in more than a single vitamin. A later group of test animals was therefore fed the deficiency diets fortified (per kg. of diet) as follows: thiamine, 60 mg.; riboflavin, 100 mg.; pantothenic acid, 600 mg.; pyridoxine,

30 mg.; choline, 2 gm.; inositol, 600 mg.; PABA, 600 mg.; nicotinic acid, 200 mg.; biotin, 1 mg.; and folic acid, 10 mg., except that the deficient vitamin in question was omitted in each case when appropriate. Strain O rats on this supplement are designated hereinafter as "O+9V" and strain H as "H+9V". The nature of other special supplements used is indicated in the discussion of the results.

TABLE I

Tabulation of Composition of Single Vitamin Deficiency Diets

Component of Diet	Vitamin Deficient from Diet						
	Vitamin A (8)	Thiamine (9)	Riboflavin (10)	Pantothenic Acid (11)	Vitamin B_6 (12)	Choline (13)	Biotin (14)
Sucrose %	----	----	----	68%	68%	68%	73%
Cornstarch (Argo) %	65%	----	63%	----	----	----	----
Dextrinized rice starch %	----	64%	----	----	----	----	----
Casein* %	18%	13%	18%	18%	18%	17.7%††	8%
Egg white† %	----	----	----	----	----	----	10%
Crisco %	----	----	3%	8%	8%	----	----
Wesson oil %	5%	----	----	----	----	----	5%
Lard %	----	----	----	----	----	10%	----
Salts‡ %	4%	3%	4%	4%	4%	4%	4%
C.L.O.§ %	----	1%	2%	2%	2%	----	‖
Cystine %	----	----	----	----	----	0.3%	----
Yeast¶ %	8%	20%**	----	----	----	----	----
Thiamine mg./100g. diet	----	----	.20	.20	.20	.20	.20
Riboflavin mg./100g. diet	----	----	----	.20	.30	.20	.20
Niacin mg./100g. diet	----	----	2.50	----	2.50	----	----
Choline mg./100g. diet	----	----	200.	----	200.	----	100.
Calcium pantothenate mg./100g. diet	----	----	250.	----	250.	.20	1.00
Pyridoxine mg./100g. diet	----	----	.30	.20	----	.20	.20

*Borden's Labco Brand Vitamin Free.
†Palpable desiccated powdered egg white.
‡GBI Salt Mixture No. 2.
§Rexall, 2000 USP units Vitamin A and 200 USP units Vitamin D per gram.
‖Two drops Navitol with Viosterol per rat per week (3300 USP units Vitamin A, 660 units Vitamin D).
¶Anheuser-Busch, Primary Dried Yeast, Strain G.
**Autoclaved six hours.
††Extracted four times for two hours each time with boiling 95% ethanol.

III. *Results*

Because of the complexity of the results obtained and the large number of individual animals used, it is most convenient to present the results for each deficiency study separately, noting at the same time any evidences of efficacy of the vitamin in question in treating animals not responding to other vitamin supplements. Each of the

TABLE II

*Summary of Experimental Data on Alcohol Consumption of Rats Fed Vitamin A Deficient Diet**

(Alcohol consumption in ml. alcohol/100 gm. rat/day)

Variable	Strain "O"	Strain "H"	Total All Rats
No. of animals in original group	9	9	18
No. of animals completing expt.	5	3	8
Initial consumption level	0.27 (0.01–0.42)	0.08 (0.00–0.33)	0.18 (0.00–0.42)
Level at initiation of treatment	0.57 (0.50–0.62)	0.74 (0.59–0.85)	0.63 (0.50–0.85)
Rats responding to deficient vitamin	1/5	2/3	3/8
Mean drop in intake due to administering Vitamin A	0.37 ------	0.29 (0.29–0.43)	0.32 (0.29–0.43)
Rats responding to 10 B vitamins	5/5	3/3	8/8
Mean drop in intake due to 10 B	0.36 (0.23–0.54)	0.28 (0.20–0.41)	0.34 (0.20–0.54)
Rats responding to 12 vitamins	3/5	1/2	4/7
Mean drop in intake due to 12 V	0.42 (0.24–0.57)	0.23 ------	0.37 (0.23–0.57)
Rats responding to vitamin B_{12}	0/5	2/2	2/7
Mean drop in intake due to B_{12}	------ ------	0.10 (0.09–0.13)	0.10 (0.09–0.13)
Rats responding to protein	2/2	1/1	3/3
Mean drop in intake due to protein	0.39 (0.32–0.45)	0.30 ------	0.36 (0.30–0.45)
Rats responding to nucleic acid	2/2	------	2/2
Mean drop in intake due to nucleic acid	0.20 (0.20–0.20)	------ ------	0.20 (0.20–0.20)
Rats responding to amino acids	1/1	1/1	2/2
Mean drop in intake due to amino acids	0.38 ------	0.44 ------	0.41 (0.38–0.44)
Rats responding to protein-nucleic acid-amino acids	3/3	1/2	4/5
Mean drop in intake due to protein-nucleic acid-amino acids	0.29 (0.13–0.46)	0.42 ------	0.32 (0.13–0.46)
Rats responding to continuous 15 vitamins	4/4	1/2	5/6
Mean drop in intake due to continuous 15 vitamins	0.26 (0.09–0.51)	0.67 ------	0.37 (0.09–0.67)
Average total days on test diet	378 (340–410)	349 (251–412)	367 (251–412)
Average final level of alcohol intake	0.29 (0.04–0.52)	0.22 (0.09–0.32)	0.27 (0.04–0.52)

*It should be noted in Tables II to VI that the data further down in each table apply to rats which have not been completely "cured" by the preceding treatment indicated. Rats on a deficiency diet which were supplied with the missing vitamin were continuously supplied thereafter. Similarly after 10 B vitamins were given, the animals were continually supplied with abundant amounts, etc. Rats to which one vitamin was administered may have been affected 100%, yet the alcohol consumption of several may have remained at an appreciable level so that they were suitable objects for further study. Due to the complicated nature of the experiment and the fact that revision of plans was required after preliminary results had been obtained, it is impossible in tables to give the exact history of each individual rat.

seven deficiency diets was studied separately. Diagrammatic representation of individual drinking patterns are presented only to illustrate specific effects, since the patterns in general follow closely upon those previously presented (1). In the diagrams, the responses in terms of alcohol intake per day are averages of five day periods, and the data so obtained were grouped to provide the tabular material. For each variable, the mean response and the ranges seen in the test group are reported, as well as the proportion of the test group giving any response to the indicated supplement. The vast amount of data thus grouped indicates both the nature of the general responses and also the extreme individual variability which was evident.

Effects of Vitamin A Deficiency on Alcohol Consumption.—A study of the data in Table II indicates that vitamin A deficiency does not promote high levels of alcohol consumption in rats, and that vitamin A is not strikingly effective in decreasing alcohol intake levels. The data do indicate that the "vitamin A deficient diet" is inadequate in regard to its content of the B vitamins, and at least suggest the desirability of higher protein-nucleic acid levels in this particular deficiency diet. In no animal throughout the study did there occur a single large drop in alcoholic intake such as occurred when other vitamin deficiencies were corrected in separate studies. Three of eight test animals showed decreased intakes of only 0.29, 0.37, and 0.43 ml. of alcohol per 100 gm. rat per day. The "index of response" (mean response $\times$ fraction responding $\times$ 10) was thus 11.3. According to this imperfect measure, vitamin A is one third as important as the next most effective vitamin. It is therefore concluded that vitamin A may be a factor but is not a major one in the etiology of alcohol consumption in rats.

Effects of Thiamine Deficiency on Alcohol Consumption.—A study of the data in Table III indicates that a thiamine deficiency produces in most rats a higher level of alcohol intake, and that thiamine administration is able to bring about a decided lowering of this level. An example of the responses obtained is that shown in Figure I. In this animal, a secondary rise occurred after about a month, reaching a level somewhat lower than that of the intake before treatment. This rise was refractory to thiamine and disappeared only after continued treatment with vitamin B_{12}. It has been observed that in many animals on deficient diets, supplying the deficient vitamin diminishes the alcohol intake, but is ineffective in eliminating a subsequent increase in the alcohol consumption. Some entirely unrelated vitamin may be effective in this case, but it in

TABLE III

*Summary of Experimental Data on Alcohol Consumption of Rats Fed Thiamine Deficient Diet**

(Alcohol consumption in ml. alcohol/100 gm. rat/day)

Variable	Strain "O"	Strain "O" + 9V	Total Strain "O"	Strain "H"	Total All Rats
No. of animals in original group	9	10	19	9	28
No. of animals completing expt.	7	6	13	7	20
Initial consumption level	0.31	0.18	0.26	0.13	0.58
	(0.01–0.67)	(0.09–0.26)	(0.01–0.67)	(0.00–0.41)	(0.00–0.67)
Level at initiation of treatment	0.76	0.93	0.85	1.18	0.95
	(0.44–1.06)	(0.61–1.33)	(0.44–1.33)	(0.59–2.00)	(0.44–2.00)
Rats responding to thiamine	6/9	8/9	14/18	5/8	19/26
Mean drop in intake due to thiamine administration	0.43	0.63	0.55	0.66	0.58
	(0.15–0.79)	(0.32–1.05)	(0.15–1.05)	(0.14–1.23)	(0.14–1.23)
Rats responding to 10 B vitamins	9/9	------	9/9	6/8	15/17
Mean drop in intake due to 10 B	0.52	------	0.52	0.44	0.49
	(0.31–1.00)	------	(0.31–1.00)	(0.09–0.85)	(0.09–1.00)
Rats responding to 12 vitamins	2/5	------	2/5	4/5	6/10
Mean drop in intake due to 12 V	0.48	------	0.48	0.43	0.45
	(0.26–0.70)	------	(0.26–0.70)	(0.28–0.50)	(0.26–0.70)
Rats responding to vitamin B_{12}	3/4	------	3/4	5/7	8/11
Mean drop in intake due to B_{12}	0.17	------	0.17	0.28	0.24
	(0.10–0.21)	------	(0.10–0.21)	(0.11–0.43)	(0.10–0.43)
Rats responding to protein	1/1	------	1/1	1/3	2/4
Mean drop in intake due to protein	0.25	------	0.25	0.20	0.23
	------	------	------	------	(0.20–0.25)
Rats responding to nucleic acid	1/2	------	1/2	------	1/2
Mean drop in intake due to nucleic acid	0.32	------	0.32	------	0.32
	------	------	------	------	------
Rats responding to amino acids	1/1	------	1/1	1/1	2/2
Mean drop in intake due to amino acids	0.12	------	0.12	0.21	0.17
	------	------	------	------	(0.12–0.21)

Rats responding to protein-nucleic acid-amino acids	0/3	———	0/3	4/5	4/8
Mean drop in intake due to protein-nucleic acid-amino acids	———	———	———	0.25 (0.23–0.30)	0.25 (0.23–0.30)
Rats responding to continuous 15 vitamins	7/7	6/6	13/13	6/7	19/20
Mean drop in intake due to continuous 15 vitamins	0.57 (0.20–1.34)	0.32 (0.20–0.63)	0.45 (0.20–1.34)	0.42 (0.10–0.64)	0.44 (0.10–1.34)
Average total days on test diet	375 (320–410)	292 (276–302)	334 (276–410)	396 (386–410)	354 (276–410)
Average final level of alcohol intake	0.22 (0.05–0.49)	0.35 (0.12–0.82)	0.28 (0.05–0.82)	0.35 (0.06–0.67)	0.31 (0.05–0.82)

*See footnote p. 118.

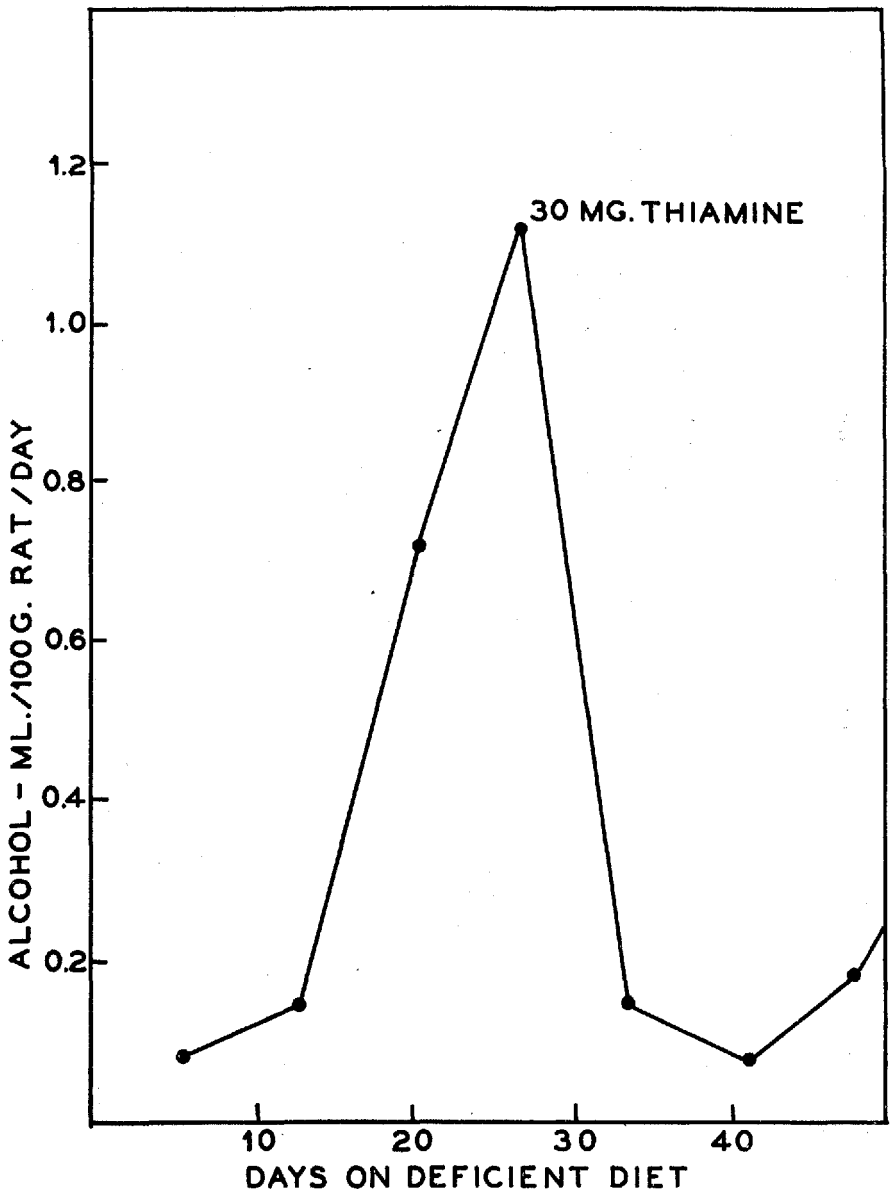

FIGURE I

Alcohol Consumption of a Thiamine Deficient Rat and the Effect of Thiamine Administration.

turn may be ineffective in controlling still subsequent increase, etc. It is therefore concluded that in these cases, a number of different deficiencies are developing at different rates, depending upon the individual vitamin requirements of the animal in question. If for example, an animal on a single deficiency diet has a high requirement for some other vitamin two deficiencies may occur at the same time, and supplying the single missing vitamin may be ineffective, unless the secondary deficiency is also corrected. In nearly every case where no response was obtained from thiamine administration to thiamine deficient rats a directly subsequent administration of nine other B vitamins along with the thiamine was effective. If secondary and tertiary deficiencies develop at slower rates, they are manifest in the data as succeeding rises in the alcohol consumption which are refractory to the vitamin previously administered.

Nineteen of twenty-six experimental animals studied responded to thiamine administration with average drop in alcohol intake of 0.58 ml. The "index of response" as calculated above was thus 42.3, second only to that of riboflavin (below). As with vitamin A, there appeared to be little significant difference between the two strains of rats. The response of strain H on the deficient diet was apparently greater than that of strain O to the protein-amino acid-nucleic acid supplement. From these data it seems inescapable that thiamine is involved in some critical fashion with the development of the appetite for alcohol in rats.

Effects of Riboflavin Deficiency on Alcohol Consumption.—A study of the data in Table IV shows that a riboflavin deficiency is highly effective in most rats in bringing about a considerable increase in the appetite for alcohol, and that riboflavin administration is effective in bringing about a prompt drop in the alcohol intake. Eighteen of a total of twenty-three rats responded to the riboflavin, with an average drop in alcohol intake of 0.90 ml! The "index of response" was thus 70.2, the highest obtained for any of the vitamins studied. This may be attributed in part to the degree of deficiency of the riboflavin deficient diet. Figure II illustrates a drop typical of those obtained from the administration of riboflavin. This animal was one from "O" strain in which the deficient diet was supplemented by large quantities of the other B vitamins as previously indicated. The curve illustrates the development of successive deficiencies on a diet supposedly deficient in only one respect. In this case there is an excellent indication that the specific deficiencies which appeared to develop successively on this diet were those of riboflavin, thiamine, and vitamin B_{12}. It should be noted that each point on the graph represents a five day average.

TABLE IV

*Summary of Experimental Data on Alcohol Consumption of Rats Fed Riboflavin Deficient Diet**

(Alcohol consumption in ml. alcohol/100 gm. rat/day)

Variable	Strain "O"	Strain "O" + 9V	Total Strain "O"	Strain "H"	Total All Rats
No. of animals in original group	9	10	19	15	34
No. of animals completing expt.	4	7	11	8	19
Initial consumption level	0.33 (0.07–0.81)	0.23 (0.14–0.42)	0.28 (0.07–0.81)	0.20 (0.00–0.62)	0.24 (0.00–0.81)
Level at initiation of treatment	1.02 (0.55–1.80)	1.47 (0.34–2.80)	1.32 (0.34–2.80)	1.01 (0.56–1.54)	1.17 (0.34–2.80)
Rats responding to riboflavin	3/4	7/8	10/12	8/11	18/23
Mean drop in intake due to riboflavin administration	0.86 (0.45–1.57)	1.36 (0.64–2.51)	1.21 (0.45–2.51)	0.51 (0.17–0.90)	0.90 (0.17–2.51)
Rats responding to 10 B vitamins	2/2	(6/6†)	2/2	9/12	11/14
Mean drop in intake due to 10 B	1.10 (0.70–1.50)	[(0.64†) (0.25–1.55)]	1.10 (0.70–1.50)	0.34 (0.29–0.56)	0.49 (0.29–1.50)
Rats responding to 12 vitamins	3/3	———	3/3	7/11	10/14
Mean drop in intake due to 12 V	0.40 (0.22–0.65)	———	0.40 (0.22–0.65)	0.24 (0.12–0.35)	0.28 (0.12–0.65)
Rats responding to vitamin B_{12}	3/3	2/2	5/5	9/11	14/16
Mean drop in intake due to B_{12}	0.46 (0.17–0.78)	0.33 (0.20–0.46)	0.40 (0.17–0.78)	0.49 (0.16–1.07)	0.47 (0.16–1.07)
Rats responding to protein	0/1	———	0/1	0/2	0/3
Mean drop in intake due to protein	———	———	———	———	———
Rats responding to nucleic acid	0/1	———	0/1	0/2	0/3
Mean drop in intake due to nucleic acid	———	———	———	———	———
Rats responding to amino acids	2/2	———	2/2	5/5	7/7

Mean drop in intake due to amino acids	0.37 (0.18–0.56)	....	0.37 (0.18–0.56)	0.46 (0.36–0.67)	0.43 (0.18–0.67)
Rats responding to protein-nucleic acid-amino acids	3/3	.....	3/3	7/10	10/13
Mean drop in intake due to protein-nucleic acid-amino acids	0.83 (0.56–1.20)	....	0.83 (0.56–1.20)	0.59 (0.31–1.21)	0.66 (0.31–1.21)
Rats responding to continuous 15 vitamins	4/4	.....	4/4	1/2	5/6
Mean drop in intake due to continuous 15 vitamins	0.47 (0.25–0.74)	....	0.47 (0.25–0.74)	0.55	0.49 (0.25–0.74)
Average total days on test diet	387 (368–410)	297 (294–300)	330 (294–410)	222 (171–392)	284 (171–410)
Average final level of alcohol intake	.09 (0.02–0.16)	0.28 (0.02–0.68)	0.21 (0.02–0.68)	0.20 (0.03–0.39)	0.21 (0.02–0.68)

*See footnote p. 118.
†Thiamine only.

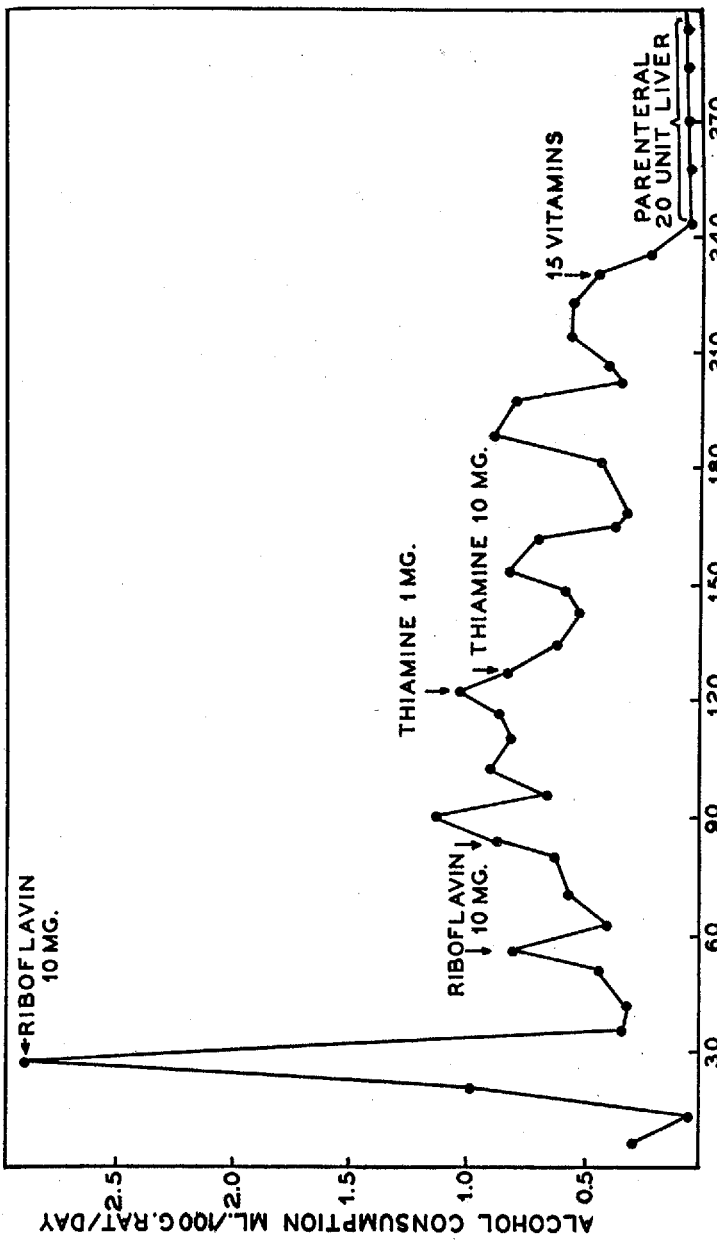

FIGURE II

Alcohol Consumption of a Riboflavin Deficient Rat.

In general the strain differences noted in the responses were appreciable but not large. It seemed that the animals on diets supplemented with large amounts of other vitamins tended to become more deficient in the one missing vitamin (as manifest by increased alcohol intake) than did animals which were on diets not so supplemented. This trend was observed in most cases throughout this study. The response to riboflavin was apparently considerably less dramatic in the strain H animals than in strain O. The data leave little question as to the importance of riboflavin in the development of the alcoholic appetite in rats.

Effects of Pantothenic Acid Deficiency on Alcohol Consumption. —An examination of Table V indicates clearly that pantothenic acid is involved in the mechanism of the development of an appetite for alcohol in rats. Fifty-two out of seventy deficient animals responded to pantothenic acid with a lowered intake of alcohol, giving an "index of response" of 38.5. A considerable strain difference was apparent. The "H" strain animals appeared not to be as deficient on the supplemented diet as the "O" strain animals, and did not therefore respond as markedly. A considerable mortality was encountered in the "O" strain before treatment was instituted, and additional data not in the table indicated that on the diet supplemented with other B vitamins, this was particularly true. A consideration of the data thus leads us to the conclusion that strain "O" has a considerably higher pantothenic acid requirement than does strain "H". A typical response of a strain "H" animal to pantothenic acid is shown in Figure III. This animal did not develop the appetite for alcohol for some time (about 100 days on the diet), differing in this manner from litter mates that showed a rise in intake in about half this period. A prompt response was obtained with a massive dose of pantothenic acid, and the intake remained at essentially zero for several months until the animal was killed. Doses of the vitamin used for most other animals were from one tenth to one third of this amount, and appeared to be equally effective.

The fact that 28 out of 30 "O" strain rats responded to pantothenic acid administration shows clearly that this vitamin is intimately concerned with the control of alcohol appetite. The fact that riboflavin caused on the average larger decreases in alcohol consumption might be interpreted as meaning merely that the deficiency diet in this case was more deficient. The relative importance of the magnitude of the consumption decrease, as compared with the proportion of the animals responding, is difficult to evaluate.

TABLE V

*Summary of Experimental Data on Alcohol Consumption of Rats Fed Pantothenic Acid Deficient Diet**

(Alcohol consumption in ml. alcohol/100 gm. rat/day)

Variable	Strain "O"	Strain "O" + 9V	Total Strain "O"	Strain "H"	Strain "H" + 9	Total Strain "H"	Total All Rats
No. of animals in original group	9	20	29	15	10	25	54
No. of animals completing expt.	4	9	13	12	10	22	35
Initial consumption level	0.31 (0.01–0.92)	0.41 (0.10–0.92)	0.38 (0.01–0.92)	0.22 (0.00–0.48)	0.27 (0.12–0.60)	0.24 (0.00–0.60)	0.31 (0.00–0.92)
Level at initiation of treatment	1.02 (0.76–1.66)	0.82 (0.60–1.29)	0.91 (0.60–1.66)	0.92 (0.30–1.42)	0.51 (0.20–1.63)	0.74 (0.20–1.63)	0.81 (0.20–1.66)
Rats responding to pantothenic acid	5/6	9/9	14/15	7/10	5/10	12/20	26/35
Mean drop in intake due to pantothenic acid	0.59 (0.13–1.64)	0.50 (0.25–1.09)	0.53 (0.13–1.64)	0.71 (0.40–0.91)	0.24 (0.12–0.36)	0.51 (0.12–0.91)	0.52 (0.12–1.64)
Rats responding to 10 B vitamins	4/4	------	4/4	10/11	------	10/11	14/15
Mean drop in intake due to 10 B	0.45 (0.35–0.55)	------	0.45 (0.35–0.55)	0.53 (0.23–1.03)	------	0.53 (0.23–1.03)	0.51 (0.23–1.03)
Rats responding to 12 vitamins	1/1	------	1/1	7/8	------	7/8	8/9
Mean drop in intake due to 12 V	0.13 ------	------	0.13 ------	0.30 (0.15–0.58)	------	0.30 (0.15–0.58)	0.28 (0.13–0.58)
Rats responding to vitamin B_{12}	------	------	------	8/8	------	8/8	8/8
Mean drop in intake due to B_{12}	------	------	------	0.29 (0.10–0.85)	------	0.29 (0.10–0.85)	0.29 (0.10–0.85)
Rats responding to protein	------	------	------	------	------	------	------
Mean drop in intake due to protein	------	------	------	------	------	------	------
Rats responding to protein-nucleic acid-amino acids	2/2	------	2/2	7/9	------	7/9	9/11
Mean drop in intake due to protein-nucleic acid-amino acids	0.39 (0.32–0.45)	------	0.39 (0.32–0.45)	0.37 (0.16–0.84)	------	0.37 (0.16–0.84)	0.37 (0.16–0.84)
Rats responding to continuous 15 vitamins	2/2	------	2/2	6/7	------	6/7	8/9
Mean drop in intake due to continuous 15 vitamins	0.22 (0.14–0.30)	------	0.22 (0.14–0.30)	0.26 ------	------	0.26 ------	0.27 (0.14–0.30)
Average total days on test diet	334 (135–410)	181 (85–300)	224 (85–410)	320 (170–405)	88 (85–100)	215 (85–405)	218 (85–410)
Average final level of alcohol intake	0.08 (0.02–0.14)	0.25 (0.08–0.51)	0.19 (0.02–0.51)	0.30 (0.07–0.52)	0.40 (0.08–1.46)	0.35 (0.07–1.46)	0.29 (0.02–1.46)

*See footnote p. 118.

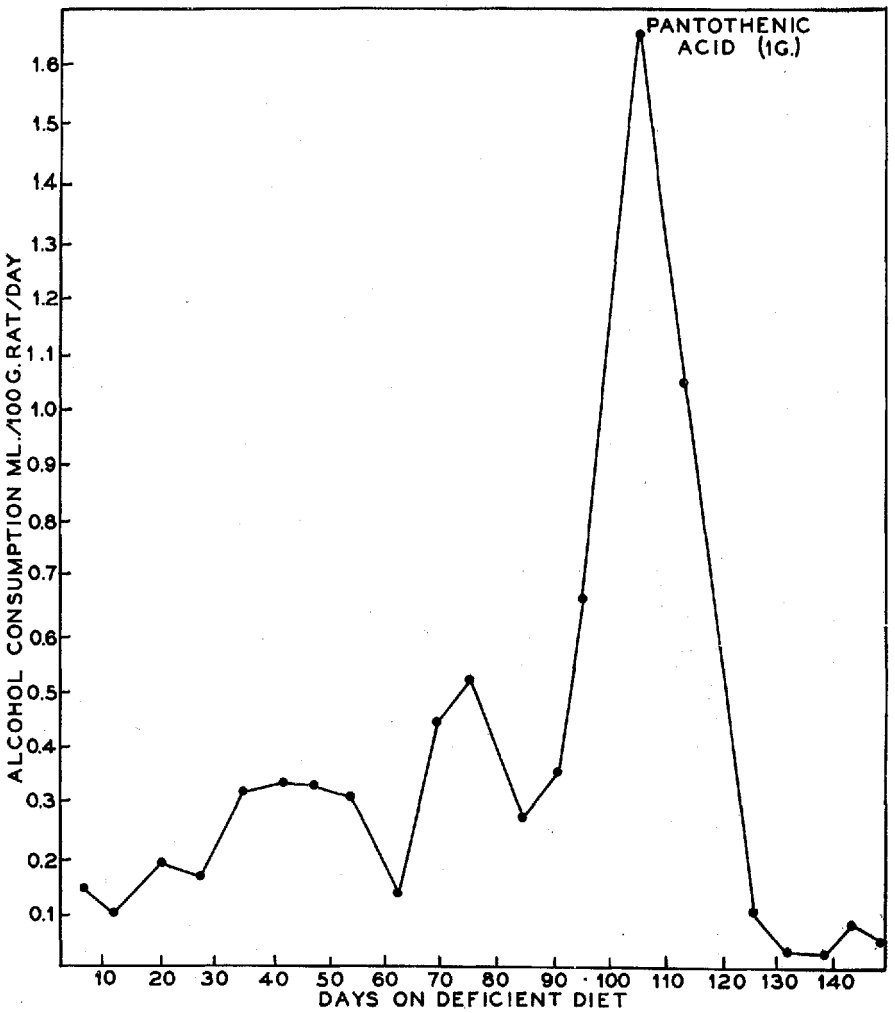

FIGURE III

Effect of Pantothenic Acid Deficiency and Supplementation on Alcohlol Consumption by a Rat.

Effect of Vitamin B_6 Deficiency on Alcohol Consumption.—The data in Table VI leave little doubt that vitamin B_6 is also involved in a definite manner in the etiology of alcoholism in rats. Twenty out of a total of thirty-three animals responded to give a mean drop of 0.56 ml. and an "index of response" of 33.6. Significant strain differences were not apparent. Figure IV illustrates a typical response to vitamin B_6 administration.

TABLE VI

*Summary of Experimental Data on Alcohol Consumption of Rats Fed Pyridoxine Deficient Diet**

(Alcohol consumption in ml. alcohol/100 gm. rat/day)

Variable	Strain "O"	Strain "O" + 9V	Total Strain "O"	Strain "H"	Total All Rats
No. of animals in original group	9	10	19	15	34
No. of animals completing expt.	4	9	13	7	20
Initial consumption level	0.35 (0.15–0.70)	0.21 (0.06–0.60)	0.28 (0.06–0.70)	0.55 (0.07–2.30)	0.40 (0.06–2.30)
Level at initiation of treatment	0.86 (0.56–1.63)	0.69 (0.45–0.98)	0.77 (0.45–1.63)	1.09 (0.53–2.43)	0.91 (0.45–2.43)
Rats responding to pyridoxine	6/9	8/10	14/19	6/14	20/33
Mean drop in intake due to pyridoxine administration	0.58 (0.38–0.91)	0.43 (0.08–0.88)	0.50 (0.08–0.91)	0.69 (0.35–1.56)	0.56 (0.08–1.56)
Rats responding to 10 B vitamins	4/5	(4/4)†	4/5	8/14	12/19
Mean drop in intake due to 10 B	0.51 (0.24–0.81)	(0.45)† (0.12–0.74)†	0.51 (0.24–0.81)	0.67 (0.41–1.20)	0.62 (0.24–1.20)
Rats responding to 12 vitamins	1/4	.....	1/4	6/13	7/17
Mean drop in intake due to 12 V	0.27	....	0.27	0.28 (0.12–0.43)	0.28 (0.12–0.43)
Rats responding to vitamin B_{12}	1/1	.....	1/1	8/9	9/10
Mean drop in intake due to B_{12}	0.10	....	0.10	0.39 (0.18–0.90)	0.36 (0.10–0.90)
Rats responding to protein	.....	.....	.....	1/2	1/2
Mean drop in intake due to protein	....	....	....	0.20	0.20 .....
Rats responding to nucleic acid	.....	.....	.....	3/4	3/4
Mean drop in intake due to nucleic acid	....	....	....	0.30 (0.18–0.47)	0.30 (0.18–0.47)
Rats responding to amino acids	.....	.....	.....	0/1	0/1

Mean drop in intake due to amino acids	------	------	------	------	------
	------	------	------	------	------
Rats responding to protein-nucleic acid-amino acids	2/4	------	2/4	0/11	2/15
Mean drop in intake due to protein-nucleic acid-amino acids	0.28 (0.21–0.36)	------	0.28 (0.21–0.36)	------	0.28 (0.21–0.36)
Rats responding to continuous 15 vitamins	4/4	6/9	10/13	3/6	13/19
Mean drop in intake due to continuous 15 vitamins	0.40 (0.15–0.77)	0.43 (0.13–0.87)	0.42 (0.13–0.87)	0.23 (0.10–0.40)	0.37 (0.10–0.87)
Average total days on test diet	406 (400–412)	290 (286–294)	326 (286–412)	409 (392–431)	355 (286–431)
Average final level of alcohol intake	.10 (0.05–0.15)	0.30 (0.05–0.59)	0.24 (0.05–0.59)	0.36 (0.12–0.67)	0.28 (0.05–0.67)

*See footnote p. 118.
†Thiamine only.

In many of the deficiencies studied most of the animals tested responded by a diminished alcohol intake to an increase in the protein or amino acid content of the diet. Pyridoxine deficient rats in general did not respond to high protein diets; in fact there was often a distinct rise in alcohol consumption. This finding is in accord with the known fact that pyridoxine is required for the catabolic use of amino acids for energy purposes.

A summary of the results obtained from the five vitamins discussed is presented in Table VII in order that the responses of the various groups studied may be more readily compared.

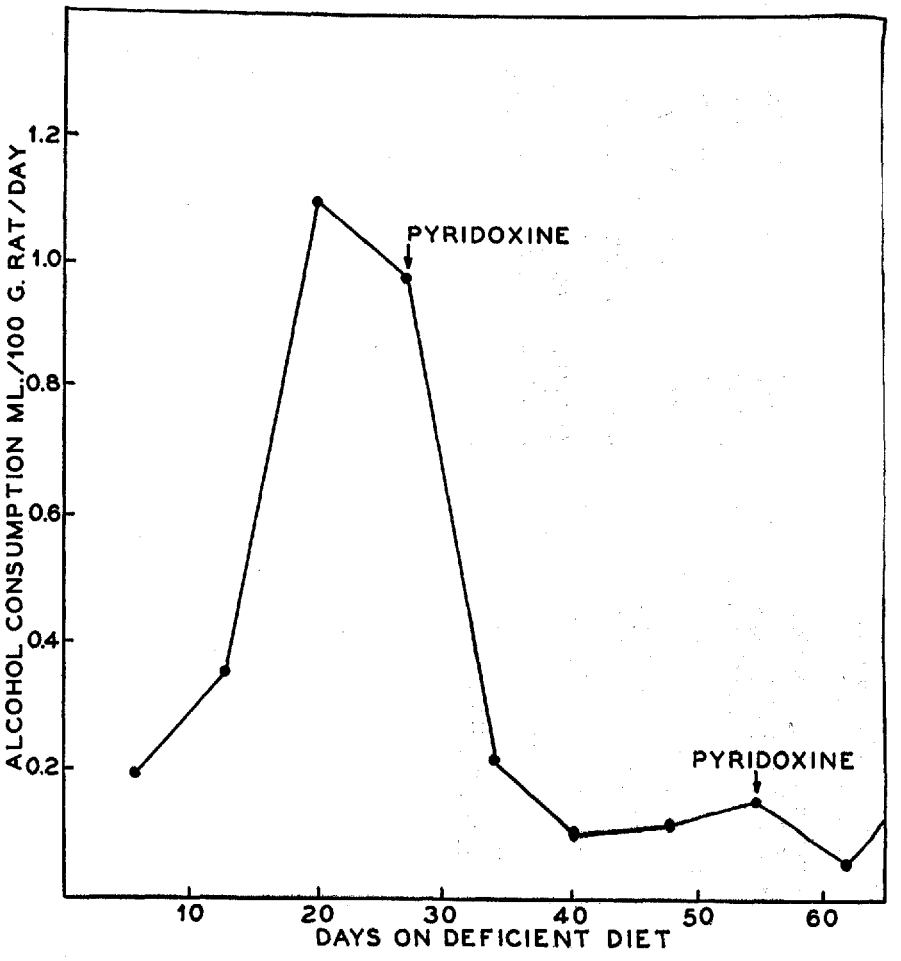

FIGURE IV

Effect on Vitamin B_6 on Alcohol Consumption by a Rat.

TABLE VII

Summary of Effect of Treatment of Single Vitamin Deficiency With Various Vitamins on Alcohol Consumption of Rats

(Alcohol consumption in ml. alcohol/100 gm. rat/day)

Strain	Variable	Vitamin A	Thiamine	Riboflavin	Pantothenic Acid	Vitamin B_6
O	Rats responding	1/5	6/9	3/4	5/6	6/9
	Mean drop in intake	0.37	0.43	0.86	0.59	0.58
	Range	-------	(0.15–0.79)	(0.45–1.57)	(0.13–1.64)	(0.38–0.91)
O + 9	Rats responding	-------	8/9	7/8	9/9	8/10
	Mean drop in intake	-------	0.63	1.36	0.50	0.43
	Range	-------	(0.32–1.05)	(0.64–2.51)	(0.25–1.09)	(0.08–0.88)
Total O	Rats responding	1/5	14/18	10/12	14/15	14/19
	Mean drop in intake	0.37	0.55	1.21	0.53	0.50
	Range	-------	(0.15–1.05)	(0.45–2.51)	(0.13–1.64)	(0.08–0.91)
H	Rats responding	2/3	5/8	8/11	7/10	6/14
	Mean drop in intake	0.29	0.66	0.51	0.71	0.69
	Range	(0.29–0.43)	(0.14–1.23)	(0.17–0.90)	(0.40–0.91)	(0.35–1.56)
H + 9	Rats responding	-------	-------	-------	5/10	-------
	Mean drop in intake	-------	-------	-------	0.24	-------
	Range	-------	-------	-------	(0.12–0.36)	-------
Total H	Rats responding	2/3	5/8	8/11	12/20	6/14
	Mean drop in intake	0.29	0.66	0.51	0.51	0.69
	Range	(0.29–0.43)	(0.14–1.23)	(0.17–0.90)	(0.12–0.91)	(0.35–1.56)
Total	Rats responding	3/8	19/26	18/23	26/35	20/33
All Rats	Mean drop in intake	0.32	0.58	0.90	0.52	0.56
	Range	(0.29–0.43)	(0.14–1.23)	(0.17–2.51)	(0.12–1.64)	(0.08–1.56)
Index of Response		11.3	42.3	70.2	38.5	33.6

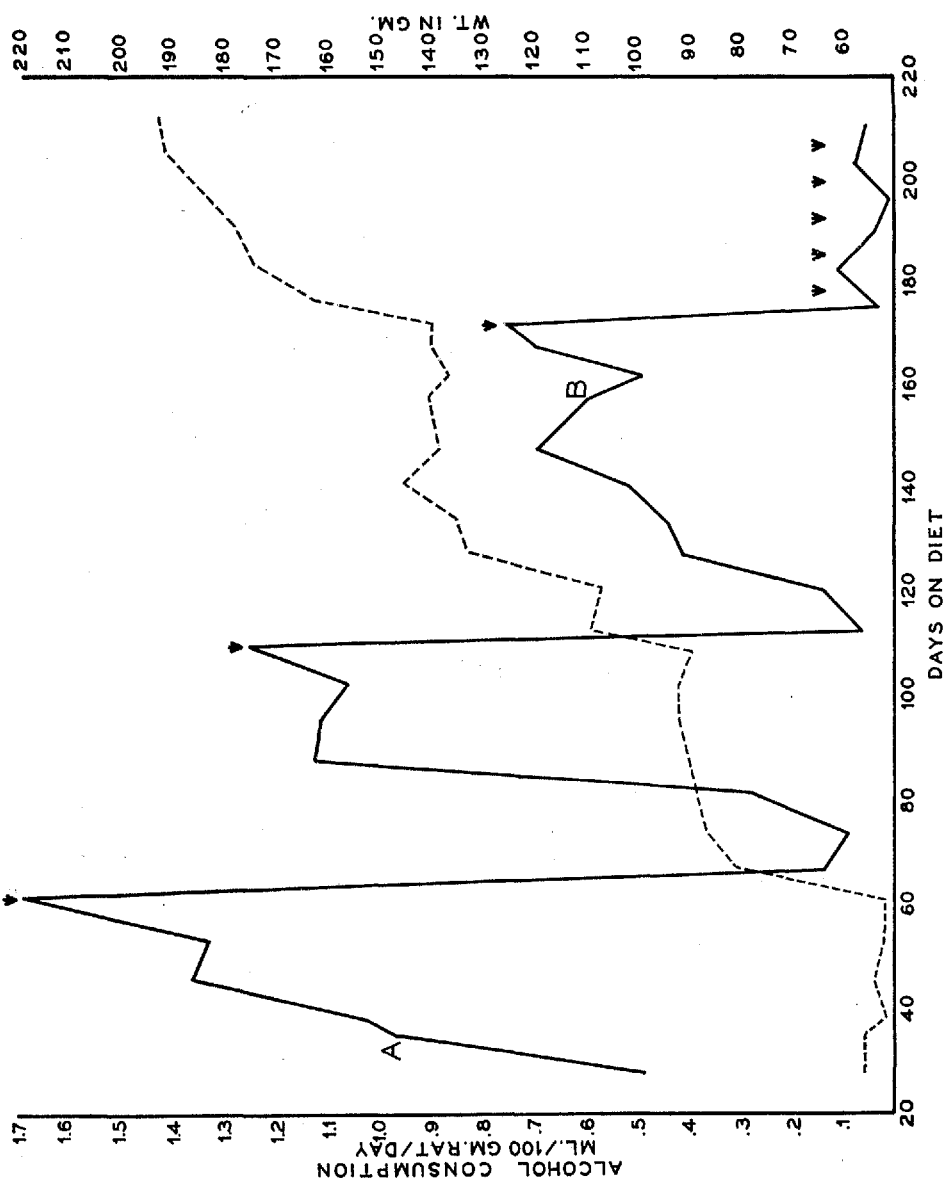

FIGURE V.

Effects of Multiple Vitamin Supplementation on Growth and Alcohol Consumption of a Rat. Solid line represents alcohol consumption; broken line represents weight of the animal. A represents the administration of desoxycorticosterone, B the administration of cortisone, and the arrows represent the administration of the 15 vitamin diet plus 20 unit liver.

Miscellaneous Effects.—Whereas there was a tendency toward increased alcohol intake in rats on choline and biotin deficient diets, addition of these vitamins to the diet and parenteral biotin therapy had little or no effect. The data do not merit presentation in detail, and suggest that *in most rats* neither biotin nor choline is intimately associated with the alcoholic appetite.

A group of fourteen rats were placed on Diet A, (3) and when their alcohol intake had arisen to an appropriate level, individual rats were treated parenterally with desoxycorticosterone, cortisone, gonadotropic hormone (Gonadophysin Searle), and 0.90% saline. In no case was a significant response elicited as indicated by alcohol consumption. Supplementation of the diet of these animals for two days with the ten vitamin supplement previously described brought about a prompt drop in alcohol intake. The intake later rose again, and could be cured again with the supplement. Figure 5 illustrates the successive "cure" of an animal three different times. The weight of the rat, which shows a very close correlation with the nutrition as judged by alcohol intake, is also included in this graph. There is therefore a strong probability that under proper conditions the alcohol consumption of rats can be used effectively to study vitamin deficiencies and in developing vitamin assay methods.

IV. *Discussion*

A large amount of experimental data has been presented to show that riboflavin, thiamine, pantothenic acid, and pyridoxine deficiencies are effective in increasing the appetite of rats for 10% alcohol, and that the addition of these vitamins to the diet of the appropriately deficient rats causes a remission of the high levels of alcohol consumption. The data bear out strongly the concept of genetotrophic disease previously presented (6). The evidence for the development of different secondary deficiencies in various individual rats emphasizes the degree to which individual vitamin requirements may vary. Although it has been inexpedient to incorporate the evidence into this paper, it would be safe to say that no two animals in this work have reacted uniformly throughout the course of the treatment. There seems little doubt that, if the requirements for each of the vitamins by a hypothetical average rat were set arbitrarily along a base line, the real requirements of individual animals would fall well above and below this line. Those animals whose requirements fall appreciably above the line suffer

mild deficiencies on so-called normal diet and exhibit these deficiencies by increased alcohol consumption.

The vitamins which have been shown to be most effective in the abolishment of alcohol appetite in rats are those which function in the early stages of carbohydrate metabolism (riboflavin, thiamine and pantothenic acid) and in the utilization of amino acids as an energy source (pyridoxine).

It seems pertinent to the subject to point out that most strong appetites about which we have some physiological understanding arise as the result of a deficiency of the element for which the appetite exists. It seems possible that the appetite for alcohol may arise as the result of its sparing action on some materials which are lacking in the body in adequate amounts. Further study of the exact modes of the metabolism of alcohol may show that as an energy source, alcohol spares the metabolic mechanisms needing the above vitamins in a manner analogous to that in which it is known to spare thiamine containing systems.

It should be pointed out, however, that in early experiments in which animals were on diets deficient in B vitamins generally (1) the administration of the four vitamins above did not "cure" the rats whereas a more complete supplement of B vitamins did. It seems that to be free from alcohol appetite, rats must have their entire metabolic machinery functioning satisfactorily but that certain deficiencies are more effective than others in promoting the appetite.

Regarding the development of the deficiency, it is of some interest to note that the four vitamins under discussion all bear hydroxyl groups which are phosphorylated in the functional form of the vitamin. Dr. Thomas Bardos of the Biochemical Institute has pointed out that Verzar and coworkers have previously shown that the phosphorylation of riboflavin is under adrenocortical control, and that a similar situation may well hold for the conversion of the other vitamins in this group to the active coenzymes. Such a consideration may help explain the data obtained by other workers indicating an interrelationship of the adrenal cortex with alcoholism.

Summary

1. Evidence is presented to show that deficiencies of thiamine, riboflavin, pantothenic acid, or pyridoxine are relatively effective in increasing the *ad libitum* consumption of 10% alcohol by

rats, but that other vitamins may also function to a lesser degree.

2. Data are presented to show that on many standard deficiency diets, some rats develop secondary and tertiary deficiencies due to individual differences in their requirements for specific vitamins.
3. The findings are interpreted in terms of the genetotrophic concept.

10 V

Code for Vitamin Supplements

	Per Day
Thiamine	0.6 mg.
Pyridoxine	0.3 mg.
Calcium Pantothenate	6.0 mg.
Riboflavin	1.0 mg.
Choline	20.0 mg.
Inositol	6.0 mg.
PABA	6.0 mg.
Nicotinic Acid	2.0 mg.
Biotin	0.02 mg.
Folic Acid	0.2 mg.

12 V

Same as 10 V with the addition of

	Per Day
Vitamin A	800 units
Vitamin D	170 units

15 V

Same as 12 V, with the addition of

	Per Day
Vitamin C	12.0 mg.
Vitamin K	0.4 mg.
Vitamin E	1.0 mg.

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XIV. Individual Excretion Patterns in Laboratory Rats

by

JANET G. REED

In order to test the validity of the tentative conclusion that individual patterns in humans exist independent of diets and environmental conditions, we have explored the possible existence of such patterns in laboratory rats when these variables are controlled. Preliminary tests were carried out with about 20 rats in order to select those which seemed to show distinct differences. It appeared from inspection of the results obtained from this larger group that each of the individual rats had a somewhat consistent excretion pattern and that some were more distinctive than others when considered from the standpoint of a limited number of measurements made. The rats used in this study were selected for further experimentation because their excretion patterns appeared to be relatively distinctive.

Observed differences in the alcoholic consumption of individual rats as well as their responses to vitamin supplements (1) have already indicated differences in metabolic patterns. A further study by this approach was underway when this investigation was started and is reported elsewhere (p. —). The present study is an outgrowth and extension of these earlier studies.

Experimental

Rats numbers 3, 5, 12 and 13 are from a highly inbred strain (brother-sister mated for 101 generations) of Wistar rats, from the Nutrition Laboratories at Iowa State College, Ames, Iowa. Rat No. 16 (47th generation of brother-sister mating) is from the Wistar W-1-B strain obtained from the Glaxo Laboratories in Middlesex, England. Rats "H" and "J" are from other colonies not highly inbred, but originally from the Wistar strain.

The rats were placed in individual false-bottom cages and given water and food (Purina dog chow) *ad lib*. Each day for a period of 7 to 11 days the rats were placed in individual metabolism cages for a few hours until an adequate volume of urine was collected. During the collection period food and water were withheld to prevent contamination of the urine sample. Immediately after collection the urine samples were frozen until analyzed.

The urine samples were analyzed for creatinine by the standard picrate colorimetric method (2), for phosphorus by the method of Fiske and Subbarow (3), and for amino acids by two-dimensional paper chromatography using the method of Berry and Cain (4). To facilitate expressing the amino acid values on a comparable basis aliquots of urine containing 50 μg. of creatinine were used as the test volume for chromatographing.

The results of these determinations are summarized in Table I which shows the average value for each rat and the standard deviations. Creatinine is expressed as mg./ml. and the values for the amino acids and phosphate are expressed as mg./mg. of creatinine in order to eliminate the effects of variability in the concentration of the urine samples. Each average is derived from at least seven samples, more often from 9 to 11.

On the amino acid chromatograms there appeared several ninhydrin positive spots the identities of which have not been estab-

TABLE I

Urinary Patterns in Laboratory Rats

	No. 3	No. 5	No. 12	No. 13	No. 16	"H"	"J"
Creatinine							
Aver.(mg./ml.)	2.04	1.76	1.87	1.62	2.25	.96	1.66
σ	.96	.91	.85	.42	.98	.62	.71
Phosphorus							
Aver.(mg./mg.Cr.)	.043	.174	.344	.463	.298	.259	.595
σ	.019	.197	.287	.179	.478	.411	.262
Aspartic Acid							
Aver.(mg./mg.Cr.)	.007	.008	.011	.015	0	.015	.013
σ	.003	.007	.007	.007	0	.008	.002
Glutamic Acid							
Aver.(mg./mg.Cr.)	.018	.037	.019	.028	.018	.017	.022
σ	.004	.036	.010	.026	.006	.008	.008
Taurine							
Aver.(mg./mg.Cr.)	.028	.044	.018	.016	.084	0	.077
σ	.031	.032	.013	.030	.043	0	.037
Alanine							
Aver.(mg./mg.Cr.)	.008	.011	.014	.014	.010	.008	.014
σ	.003	.003	.006	.005	.003	.003	.004
Citrulline							
Aver.(mg./mg.Cr.)	.007	.010	.014	.016	.012	.009	.014
σ	.003	.003	.006	.003	.000	.003	.005
Methionine Sulfoxide*							
Aver.(mg./mg.Cr.)	.013	.023	.031	.023	.026	.025	.033
σ	.007	.010	.009	.011	.012	.008	.001
Valine							
Aver.(mg./mg.Cr.)	.012	.018	.015	.016	.011	.014	.012
σ	.000	.006	.006	.006	.001	.004	.000
Leucine							
Aver.(mg./mg.Cr.)	.016	.029	.020	.027	.016	.017	.019
σ	.006	.010	.009	.007	.007	.006	.009
Lysine							
Aver.(mg./mg.Cr.)	0	.001	.007	0	.005	0	.012
σ	0	.008	.012	0	.012	0	.005

*See footnote p. 75.

listed. These spots are: 1. a purple spot with Rf value of .17, thought perhaps to be cystine; 2. a purple spot before methionine sulfoxide with an Rf value of .68; 3. an orange spot below serine with an Rf value of .15; 4. a yellow spot below serine with an Rf value of .12; 5. a blue spot appearing above valine which under ultra-violet light looks brick-red, Rf value of .71. Because of their unknown nature these spots were not quantitatively measured, but since the chromatograms were prepared on a creatinine basis, the presence or absence of the spots is considered to be of quantitative significance. The frequency of occurrence of the unknown spots in the various samples is presented in Table II.

Discussion

From inspection of Table I it may be noted that aspartic acid was always absent from the urine of rat 16, and that taurine was always missing in the case of rat "H". Lysine was absent in all samples from rats No. 3, No. 13, and "H", appeared in a few samples from rats No. 5, No. 12 and No. 16 and was present in all the samples from rat "J". Furthermore it may be noted that the relative phosphate excretions of rat "J" and No. 13 are approximately 14 and 11 times respectively that of rat No. 3. This wide variability in urinary phosphate excretion has been observed in a large number of rats in an extended study (p. 146). It may also be noted that the comparative excretion of rat "J" for most amino acids (as well as phosphate) is higher than for rat No. 3, often by two or three fold. In terms of absolute amounts of amino acids excreted per ml. of urine, rat "H" is even lower than rat No. 3.

For purposes of comparison the average of each rat was divided by the average of the group and the resultant ratios plotted in Figure 1. It appears from these graphs that rat No. 3 in most cases excretes less than the average of the group, while "J" excretes correspondingly more. Comparing rats No. 3 and "J" statistically it was found that phosphorus, aspartic acid, taurine, alanine, citrulline, methionine sulfoxide,* leucine and lysine were significantly different for the two, on the customary 5% level, while creatinine, glutamic acid, and valine were not. In like manner statistical comparison of rat No. 3 with rat No. 5 (a litter mate) showed a significant difference in the values of glutamic acid, alanine, citrulline, methionine sulfoxide,* valine, and leucine, while the differences between the creatinine, phosphorus, aspartic acid, taurine and lysine were not significant.

*See footnote p. 75.

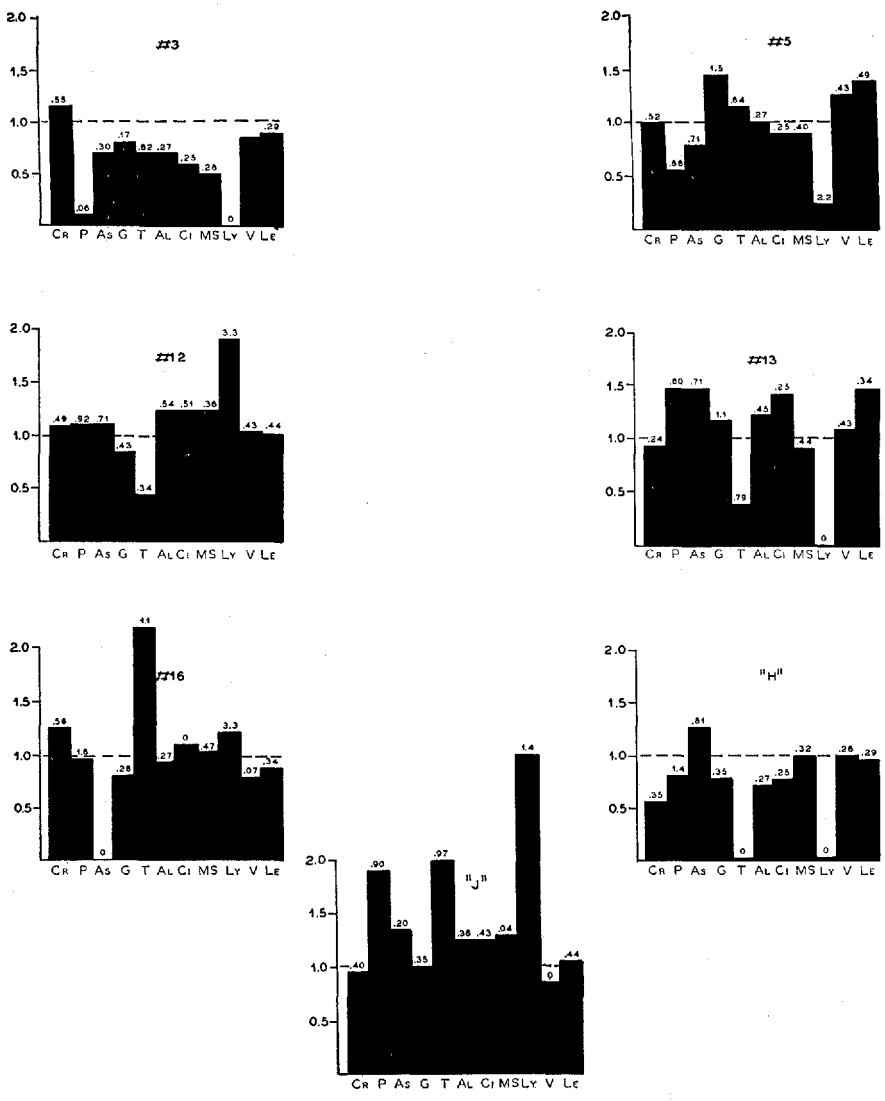

FIGURE I.

Excretion Patterns of Rats.

Vertical bars represent the ratio of the average excretion of urinary constituents (expressed as mg. constituent per mg. creatinine) for individual rats divided by average excretion for the group. The broken line indicates average excretion for the group. The number at top of bar shows standard deviation (σ) on the same scale. Cr = creatinine; P = phosphorus; As = aspartic acid; G = glutamic acid; T = taurine; Al = alanine; Ci = citrulline; MS = methionine sulfoxide*; Ly = lysine; V = valine; Le = leucine.

*See footnote p. 75.

While the above are probably extreme examples, statistical treatment of the other possible combinations indicates that a significant difference in the excretion of at least one substance exists in the case of each possible comparison.

Differences in the occurrence of the unknown spots are also apparent from inspection of Table II. Spot 1 was completely absent from the urine of rat "H", while it appeared in every sample from rats "J" and No. 16, and at least 50% of the time in daily samples from the other rats. Spot 2 never appeared in samples from rat "J", but occasionally showed up in other rats' samples. The bright orange spot, No. 3, never appeared in samples from rats No. 5, No. 12, No. 13 and "H", but did appear occasionally in the others.

TABLE II

Frequency (Percentage) of Occurrence of Unidentified Spots

	Rat No. 3	No. 5	No. 12	No. 13	No. 16	"H"	"J"
Spot No. 1	50	55	71	70	100	0	100
Spot No. 2	50	18	14	20	9	81	0
Spot No. 3	30	0	0	0	9	0	14
Spot No. 4	100	91	100	90	73	100	100
Spot No. 5	50	36	43	60	54	100	100

Conclusion

From the above results it appears that individual excretion patterns exist even in closely inbred rats on the same diet and under similar environmental conditions. While a given rat shows daily variations in its excretion pattern, the levels of excretion for most substances lie within a rather limited range. Statistically significant differences with respect to several substances may not occur in the majority of a closely inbred rat population, but the cases studied indicate that even in a highly inbred colony, each rat tends to have a pattern which is distinctive. Presumably this would be more apparent if additional items were included in the study. These findings are of course in line with what is known about genetic variability.

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XV. A Study of the Alcoholic Consumption and Amino Acid Excretion Patterns of Rats of Different Inbred Strains

by
JANET G. REED

Previous studies of the alcoholic consumption of rats have been done with animals not highly inbred and on special diets. This experiment was designed to study the alcoholic consumption of rats from highly inbred strains and on a stock diet. In addition an attempt was made to correlate metabolic patterns as evidenced in urinary studies of each strain with the alcoholic consumption of that strain.

Four strains and two substrains of rats were used for this study. They are designated as Iowa Substrain 25–32 and Iowa Substrain 65–76 (each substrain brother-sister mated 101 generations) from the Nutrition Laboratories at Iowa State College, Ames Iowa; Wistar Substrain W-1-B, Wistar Substrain W-2-A (each substrain brother-sister mated 47 generations) and Piebald (brother-sister mated 9 generations) from the Glaxo Laboratories, Middlesex, England; Fischer 344 (brother-sister mated 46 generations) from the Detroit Institute of Cancer Research, Detroit, Michigan.

Ten rats from each of the strains were placed in individual false bottom cages, given stock diet (Purina dog chow), water and 10% ethyl alcohol *ad lib*. Readings were made of the amounts of water and alcohol consumed by each rat. Alcohol consumption was expressed as milliliters of pure alcohol per 100 gram of rat per day. After a 30 day experimental period the alcohol was removed. Approximately four days after removal of the alcohol the collection of urine samples was begun. Each rat was held over a clean dry evaporating dish for a few minutes each day for a period of two to five days to collect urine samples. The daily samples for each individual rat were pooled and frozen until used.

The urine samples were analyzed for amino acids by two dimensional paper chromatography using the method of Berry and Cain (1). Aliquots of 25 μl. and 50 μl. of urine were used, but the 50 μl. sheets were beyond the range of the standards. The values obtained for the amino acids were divided by the creatinine value of the particular sample in order to eliminate as much as possible the concentration factor.

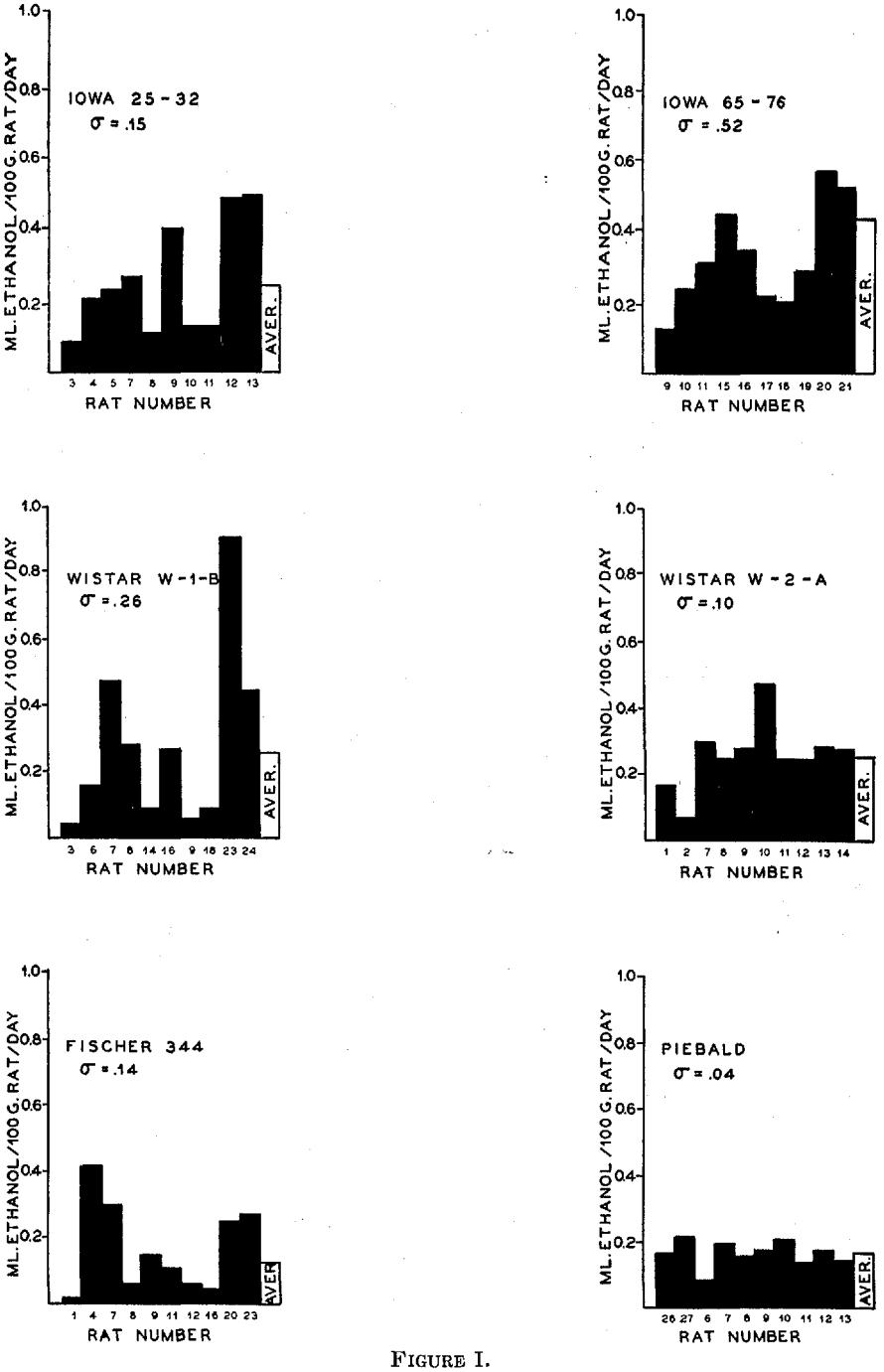


FIGURE I.

Average Daily Alcohol Consumption by Individual Rats of Six Different Strains.

The unshaded bars represent average daily alcohol consumption by all rats within each strain.

Inspection of the individual averages of alcohol consumption for the 30 day period (Figure 1) shows a wide variation within a strain. The Piebald strain, which is the most homogeneous in regard to alcohol consumption (σ=.04) is found to be significantly different (using the customary 5% level) from the Iowa 65–76 substrain and the Wistar W-2-A substrain, but is not significantly different from the others. In contrast Iowa 65–76 which is the most heterogeneous with respect to alcohol consumption (σ=.52) is significantly different from every other strain with the exception of Wistar W-1-B. It is interesting to note that the most homogeneous group (alcohol consumption) has been brother-sister mated for only 9 generations, while the most heterogeneous group on the same basis (Iowa 65–76) has been brother-sister mated for 101 generations.

Although there is a great deal of individual variation in urinary excretory products within each strain, some significant differences between the strains can be shown. The phosphate excretion (Figure 2) of the two Iowa substrains is not significantly different, nor is the phosphate excretion of the two Wistar substrains significantly different; however, the differences between the Iowa substrains and the Wistar substrains are highly significant. The Iowa substrains are also different from the Fischer and Piebald strains, although these strains are not significantly different when compared with each other or with the Wistar substrains.

It should be noted that although the rats are on identical diets there is a maximum of a six fold variation between urinary phosphate excretion of one strain compared with another. Between individual rats on identical diets there is nearly a 500 fold variation! It seems inescapable that the fecal phosphate excretion must be correspondingly high when the urinary phosphate is low but this has not been investigated.

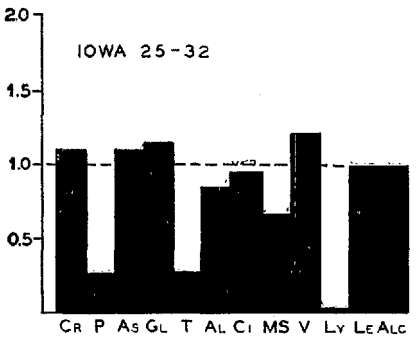
FIGURE II.

Excretion Patterns of Rats of Different Strains.

Verticle bars represent the ratios of average excretions of urinary constituents (expressed as mg. constituent per mg. creatinine) by the rats in each strain divided by the average excretion for the rats in all strains. Broken line indicates average excretion for all strains. Cr = creatinine; P = phosphorus; As = aspartic acid; Gl = glutamic acid; T = taurine; Al = alanine; Ci = citrulline; MS = methionine sulfoxide*; V = valine; Ly = lysine; Le = leucine.

Alc = average alcohol consumption by all rats in each strain expressed as ml. ethanol/100 g. rat/day.

*See footnote p. 75.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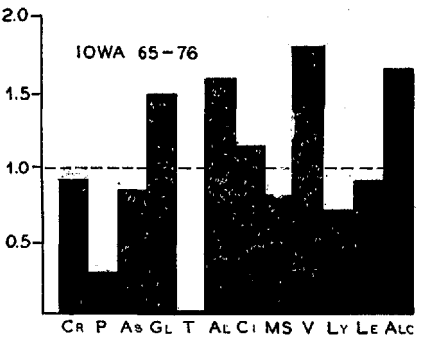
1.5
1.0
0.5
CR P AS GL T AL CI MS V LY LE ALC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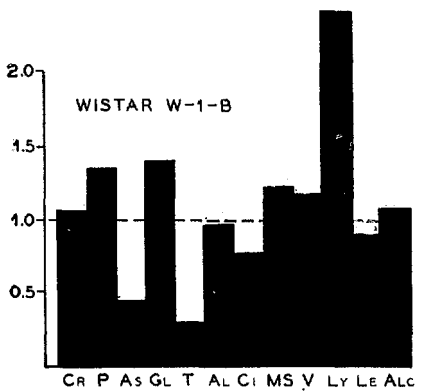
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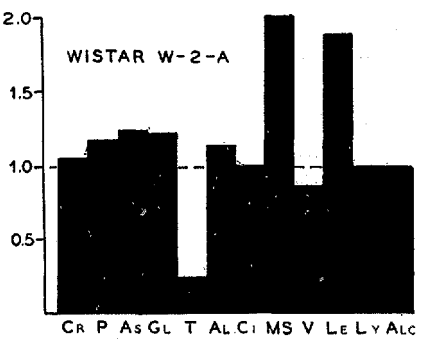
1.0
0.5
CR P AS GL T AL CI MS V LY LE ALC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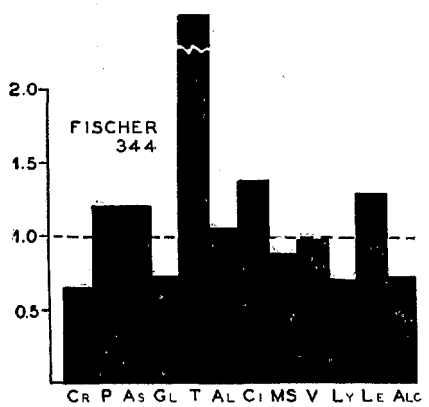
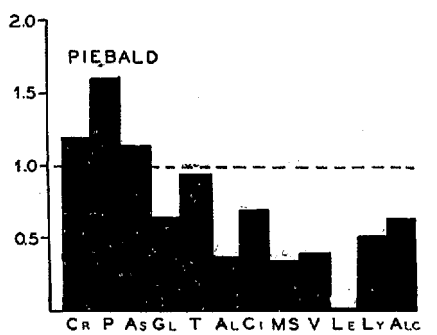
1.5
1.0
0.5
CR P AS GL T AL CI MS V LE LY ALC


FISCHER
344
2.0
1.5
1.0
0.5
CR P AS GL T AL CI MS V LY LE ALC


PIEBALD
2.0
1.5
1.0
0.5
CR P AS GL T AL CI MS V LE LY ALC

Differences in lysine excretion are apparent when Wistar W-1-B is compared with each of the other groups with the exception of its sister substrain W-2-A. In the excretion of lysine the two Iowa substrains are significantly different from each other.

Large variations in the amount of taurine excreted by the various strains are also apparent from Figure 2. The Fischer 344 strain is outstanding for the large volume of taurine excreted, while the Iowa strains excrete very small amounts. Both the Fischer 344 and Piebald strains are significantly different from the other strains in their taurine excretion.

The Wistar substrain W-2-A is significantly different from the other strains with the exception of its sister substrain W-1-B in the excretion of methionine sulfoxide.* For the excretion of practically each amino acid some statistically significant difference can be found to exist between at least two of the strains. However, the greatest strain differences appear to be concerned with the products previously discussed, namely: phosphorus, lysine, taurine, and methionine sulfoxide.*

Several spots whose identities have not been definitely established occasionally appeared on the amino acid chromatograms. They are designated as: 1. purple spot believed to be cystine, Rf .17; 2. purple spot before methionine sulfoxide, Rf .68; 3. Blue spot above valine, Rf .71; 4. orange spot below serine, Rf .15; and 5. yellow spot below serine Rf .12. The frequency of occurrence of these spots is shown in Table I.

TABLE I

Per Cent Occurrence of Unidentified Spots

Strain:	Spot No. 1	Spot No. 2	Spot No. 3	Spot No. 4	Spot No. 5
Iowa 25–32	60	80	30	0	30
Iowa 65–76	0	50	0	10	60
Wistar W–1–B	0	0	0	10	80
Wistar W–2–A	10	0	0	50	90
Fischer 344	50	10	0	0	0
Piebald	80	80	30	0	30

Any attempt to correlate the metabolic pattern of a strain or substrain with the alcohol consumption of that strain or substrain is made extremely difficult because of the great individual variation found in each group. Actually in the study of the metabolic patterns of alcoholics, no amino acid excretion figures were found to be significantly different from the controls. It was not possible to measure in rats a number of the items which were included in the

*See footnote p. 75.

study of the alcoholics because of the unavailability of rat saliva and of sufficiently large samples of rat urine.

The findings here recorded reflect the existence of a complicated genetic situation and lead to the conclusion that many genes (and other inheritance factors?) are involved in the production of distinctive urinary patterns in rats and in the determination of the appetite of the animals for alcohol. It should be theoretically possible to develop strains of animals which would have any combination of metabolic traits, but of course if they are not inbred with respect to the specific traits in question, there is no assurance that they will show uniformity with respect to these traits within the strain. It would appear that our initial observation in which we found closely inbred mice to show uniformity in their alcohol consumption was to a degree fortuitous. Among the six strains and substrains of rats used in this study only one, the Piebald, showed a high degree of uniformity in alcohol consumption.

Summary

A study of the alcohol consumption and the excretion patterns of 60 rats belonging to four strains and two substrains of highly inbred rats on identical diets indicated the existence of several striking strain differences and also highly individual differences in the animals within a strain. Strain differences involved different levels of alcohol consumption, and of phosphate, lysine, taurine and methionine sulfoxide excretion. Individual differences occurred with respect to all the items measured. No conclusions with respect to relationship between specific excretions and the appetite for alcohol could be drawn.

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XVI. A Study of the Urinary Excretion Patterns of Six Human Individuals

by

HELEN KIRBY BERRY, LOUISE CAIN, AND LORENE LANE ROGERS

As a preliminary to further investigation, the respective urinary excretion patterns of six human subjects have been explored over a period of several weeks. A limited investigation of this nature had been made previously in this laboratory (1) through the use of microbiological assay methods. With the development of paper chromatographic techniques, it was possible to analyze a given sample for a number of components in a single determination (2), and a much more extended study of urinary excretion patterns became feasible. In the present investigation each urine sample was analyzed for creatinine and glucose as well as for the amino acids that could be determined by one-dimensional paper chromatography.

Experimental

Subjects.—The individuals used in this study included two young adult females (A and B), two young adult males (C and D), and two adolescent "identical" male twins (E and F). All subjects were in good health and were on self selected diets throughout the period of investigation.

Urine samples.—Urine samples were collected daily from the two female subjects over a period of two months, from the two male adults over a period of one month, and from the twins over a period of two weeks. Because of the difficulty of collecting 24-hour samples during an extended period of time, only morning samples were used in this study. Of course there was the problem of the comparison of results due to the variability in urine concentration observed in different individuals as well as in the same individual at different times. In order to minimize these concentration effects, all results have been expressed as milligram of constituent per milligram of creatinine as is discussed below.

Determination of urinary constituents.—Creatinine was determined by a standard colorimetric method (3). A measure of total carbohydrate (which we have termed glucose) was obtained using the colorimetric procedure described by Morris (4). Amino acids were separated on one dimensional paper chromatograms and quantitatively estimated by the spot comparison method (5).

Results and Discussion

The statistically summarized results of assays are shown in Table I. It will be noted that certain of the averages and standard deviations are reported as ranges. This means of designation is used in recognition of the fact that concentrations too small to be detected by the methods employed were not necessarily zero. In cases where as many as 10% of the determinations of a given constituent for a given individual resulted in values of zero (*i.e.* less than the minimum detectable quantity), averages and standard deviations have been calculated on two bases: first, assuming the zero values to be actually zero, and second, assuming them to

TABLE I

Individual Urinary Excretion Patterns

	Female A	Female B	Male C	Male D	Twin E	Twin F
Creatinine						
No. samples	54	58	24	25	12	14
Avg. (mg./ml.)	0.82	1.60	1.58	1.57	1.23	0.98
Std. Dev.	0.47	0.78	0.52	0.42	0.53	0.45
Range	0.26–3.24	0.16–3.23	0.47–2.45	0.78–2.47	0.50–2.20	0.36–2.00
Glucose						
No. samples	46	46	24	25	8	7
Avg. (mg./mg.Cr.)	0.53	0.41	0.41	0.48	0.57	0.46
Std. Dev.	0.25	0.11	0.14	0.17	0.19	0.14
Range	0.21–1.32	0.26–0.61	0.20–0.87	0.28–0.99	0.35–0.92	0.34–0.68
Alanine						
No. samples	54	58	23	25	12	14
Avg. (mg./mg.Cr.)	0.084	0.13	0.065	0.054	0.064	0.041
Std. Dev.	0.044	0.085	0.031	0.031	0.034	0.022
Range	0.03–0.21	0.02–0.50	0.00–0.16	0.01–0.13	0.03–0.16	0.01–0.10
Glycine						
No. samples	54	58	24	25	12	14
Avg. (mg./mg.Cr.)	0.18	0.21	0.045	.027– .030	0.052	0.041
Std. Dev.	0.094	0.14	0.022	.021– .024	0.036	0.018
Range	0.00–0.48	0.04–0.68	0.00–0.16	0.00–0.08	0.00–.14	0.01–0.08
Serine						
No. samples	54	58	23	25	12	14
Avg. (mg./mg.Cr.)	.071– .074	0.14	0.045	.025– .028	0.056	0.034–0.035
Std. Dev.	.051– .055	0.092	0.024	.019– .022	0.019	0.019–0.022
Range	0.00–0.28	0.00–0.50	0.00–0.10	0.00–0.08	0.02–0.08	0.00–0.08
Glutamic Acid						
No. samples	42	54	24	25	12	14
Avg. (mg./mg.Cr.)	.034– .035	.013– .014	.012– .019	.026– .030	0.017–0.021	0.012–0.019
Std. Dev.	.050– .051	.027– .028	.025– .027	.018– .022	0.011–0.016	0.017–0.021
Range	0– .282	0– .159	0.00–0.12	0.00–0.06	0.00–0.04	0.00–0.06
Lysine						
No. samples	54	58	24	25	12	13
Avg. (mg./mg.Cr.)	.132– .134	.007– .008	.035– .042	0– .0005	.014– .015	.012– .013
Std. Dev.	.085– .089	.017– .018	.028– .032	0	.011– .012	.009– .010
Range	0.00–0.29	0– .073	0.00–0.12	0– .0005	0– .032	0– .032
pH						
No. samples	52	50				
Avg.	6.85	5.69				
Std. Dev.	0.78	0.57				
Range	5.3–8.8	5.2–7.7				

be equal to the minimum detectable quantity. The results of these two methods of calculation represent the range reported in Table I.

Constituents other than creatinine are, as previously noted, expressed as milligrams of constituent per milligram of creatinine. This procedure was adopted as the best substitute for 24-hour samples in minimizing the effect of the rather wide variations in urine concentration. The choice of creatinine as a reference substance was based in part on the fact that, in our experience, it has shown less variation between individuals than any other substance studied. This is in line with general experience. Of the four adult subjects studied here, the average creatinine excretion for three of them, on a volume basis, is practically identical.

Further evidence that data from morning samples, expressed as creatinine ratios, may be considered comparable to the results of analyses on 24-hour samples is presented in Table II. The average creatinine ratios for four amino acids determined by analysis of approximately 50 morning samples are compared with the average amino acid/creatinine ratios from seven 24-hour samples obtained from the same subject during the same period. An examination shows that the two sets of data are substantially alike.

TABLE II

Urinary Excretion Pattern of Subject A

Constituent	Based on Approx. 50 Morning Samples	Based on Seven 24-hour Samples
Creatinine (mg./ml.)	0.82	0.95
Alanine (mg./mg. Cr.)	0.084	0.053
Glycine (mg./mg. Cr.)	0.18	0.14
Serine (mg./mg. Cr.)	0.072	0.072
Lysine (mg./mg. Cr.)	0.13	0.11

The urinary excretion patterns of the individuals studied are summarized in graphic form in Fig. 1. The broken line on each profile represents the group average excretion for each substance in question, and the vertical blocks plot on the same scale the ratio of the individual averages to that of the group average for each of the individuals studied. Even a cursory glance at Fig. 1 shows

that the excretion profiles of the identical twins, E and F, are much more nearly alike than those of any two other individuals studied. According to current ideas in the field of biochemical genetics similar excretion patterns would be expected from identical twins.

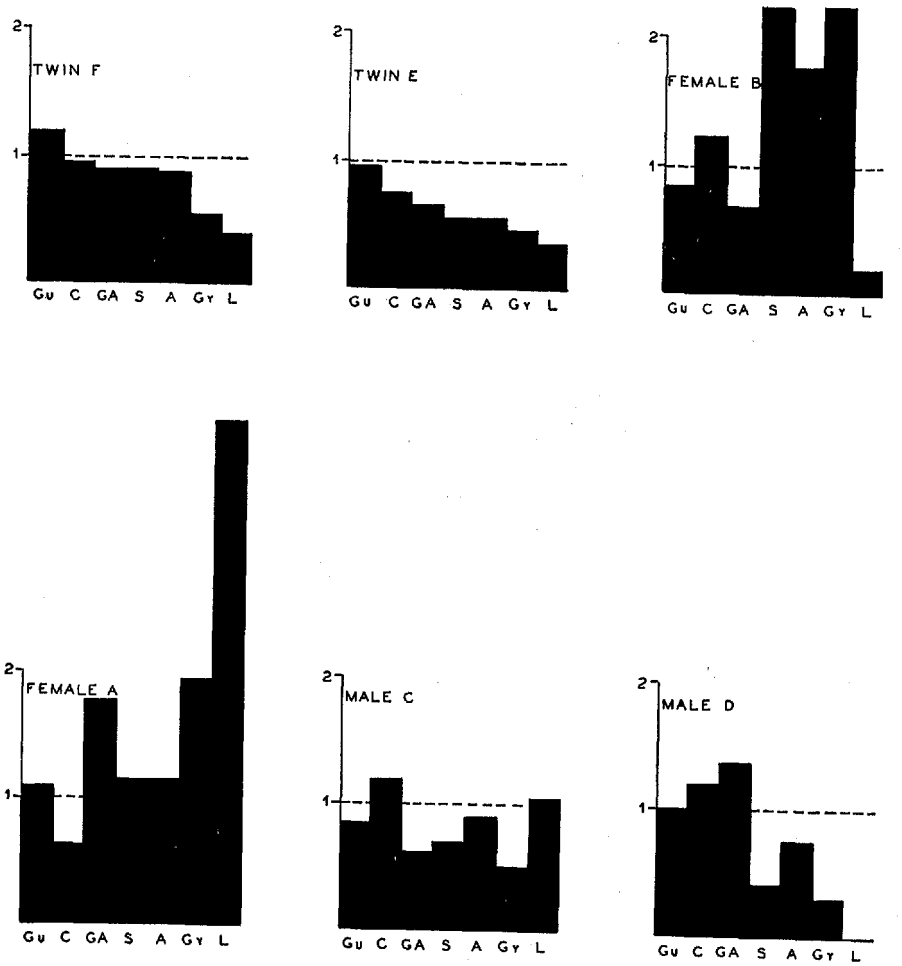

FIGURE I.

Urinary Excretion Patterns of Six Individuals.
G = glucose; C = creatine; GA = glutamic acid; S = serine: A = alanine; Gy = glycine; L = lysine. Broken line represents group average excretion (mg. constituent per mg. creatinine), and vertical bars represent ratio of individual average to group average.

The differences in the excretion patterns of various individuals other than the twins are quite striking. One of the most interesting

of these differences is that of lysine excretion. Subject A consistently excreted a relatively high concentration of lysine while subject D never excreted any detectable amount throughout the period of investigation. All other individuals studied excreted a much lower average amount of lysine than did subject A.

Comparison of the amino acid excretion rates of the male subjects with those of the female subjects reveals a consistently higher rate of excretion by the females. This sex difference has been previously observed in total α-amino nitrogen excretion as determined on single 24-hour samples (6), and is supported by a great bulk of additional data which has been gathered in our laboratories in connection with other studies.

There was some indication of cyclic variations in the female excretion patterns coincident with the menstrual cycle. The most obvious example was in the *p*H values which exhibited pronounced maxima during the menstrual period. Subject B exhibited minima in creatinine and glucose excretion at the onset of menstruation, with corresponding maxima in the amino acid/creatinine ratios. The effect was of only one or two days duration and was not observed at all in the case of subject A.

The differences in the average excretion values reported for the various individuals in Table I have been treated statistically, using the "t" test for significance (7). The results of these calculations are recorded in Table III. The numbers recorded represent minimum values for the percentage probability that the difference observed is real and not due to random fluctuations. Thus, a value of 99 recorded in Table III represents a greater than 99% probability that the difference in question is significant; a value of 98 represents a greater than 98% (but less than 99%) probability that the difference is significant, etc. All values below 90 are simply recorded as such. In cases where the excretion averages are reported in Table I as ranges, the "t" test was applied to the difference in the maximum values as well as to the difference in the minimum values, and the results are reported in Table III as a range of probabilities.

That there are significant differences in the individual excretion patterns is evident from Table III. The question arises, of course, as to the extent to which the use of creatinine as a reference substance contributes to these observed differences; *i.e.*, to what extent are the differences in creatinine ratios attributable to variations in creatinine excretion. A careful consideration of the data has clearly indicated that the differences are attributable primarily to the

urinary constituents other than creatinine. Comparisons between individuals of the same sex do not show one subject consistently excreting more of all of the amino acids than the other subject, as would be the case if differences in creatinine excretions were primarily responsible for the differences in the creatinine ratios.

TABLE III

Statistical Significance of Differences in Urinary Excretion Patterns
Per Cent Probability of Significance as Measured by "t" test (7)

	Comparison of Females	Comparison of Males	Intercomparison of Females and Males				Comparison of Twins
	A vs. B	C vs. D	A vs. C	A vs. D	B vs. C	B vs. D	E vs. F
Creatinine (mg./ml.)	99	< 90	99	99	< 90	< 90	< 90
Glucose (mg./mg.Cr.)	99	< 90	95	< 90	< 90	90	< 90
Alanine (mg./mg.Cr.)	99	< 90	90	99	99	99	90
Glycine (mg./mg.Cr.)	< 90	95–98	99	99	99	99	< 90
Serine (mg./mg.Cr.)	99	98–99	95–98	99	99	99	98–99
Glutamic Acid (mg./mg.Cr.)	99	<90–>90	<90–>90	< 90	< 90	95–98	< 90
Lysine (mg./mg.Cr.)	99	99	99	99	99	90–95	< 90
pH	99						

It is of course impossible to present in a short space a complete picture of the extensive data collected, and average excretion values covering an extended period do not reveal all that is significant. For example the average alanine excretions (per mg. of creatinine) of the two adult females were not very far apart, but the day to day graphs showed a marked dissimilarity. For one individual the the excretion was relatively uniform from day to day; for the other there were tremendous fluctuations which appeared consistently for weeks. Differences such as these (and the case cited was by no means unique) appear to be distinctive and a part of the "pattern" of the individual concerned, but do not appear in the tabulations. A separate study of these fluctuations is being made.

It may also be noted that during the course of this study new solvents and developing agents were tried and chromatographic spots corresponding to unknown substances were discovered. Many additional observations indicative of individuality of excretion patterns were made which are not recorded specifically here.

The extent to which differences in diet may have influenced the results of the present investigation cannot be assessed with certainty. It seems likely, however, that in experiments of such long duration many dietary effects would be eliminated. It would appear that the pronounced and consistent sex differences observed cannot be attributed to differences in diet. Other studies bearing on the effects of diet are reported elsewhere in this Bulletin.

Summary

Creatinine, glucose, alanine, glycine, serine, glutamic acid, lysine, and *p*H have been determined on consecutive morning urine samples from six subjects over a period of from two weeks to two months. The subjects all possess excretion patterns which are significantly distinct from each other. Amino acid excretion is consistently higher in the female subjects than in the male. The urinary excretion patterns of a pair of identical twins show greater similarities than do the patterns of any of the other subjects investigated.

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XVII. Further Studies on Individual Urinary and Salivary Amino Acid Patterns

by

HELEN KIRBY BERRY

In a series of exploratory studies such as are described in this bulletin, it has not been feasible to do detailed advance planning because, since the work was pioneering in nature, it was impossible to tell in advance of the actual work what particular lines of study would develop the greatest interest.

In the present article there are set forth results from various studies which have a bearing on individuality in urinary excretion patterns and in saliva composition.

Two dimensional urine chromatograms.—In Figures 1–10 are presented diagrams depicting comparatively the urinary excretion patterns of 10 young healthy individuals, 2 boys, 2 girls, 5 adult males, 1 adult female, picked from a large group to show differences and similarities. These individuals were not under dietary control. The figures are drawn to scale representing the size of the amino acid spots obtained from samples of urine containing 40 micrograms of creatinine (20–75 microliters). Each figure

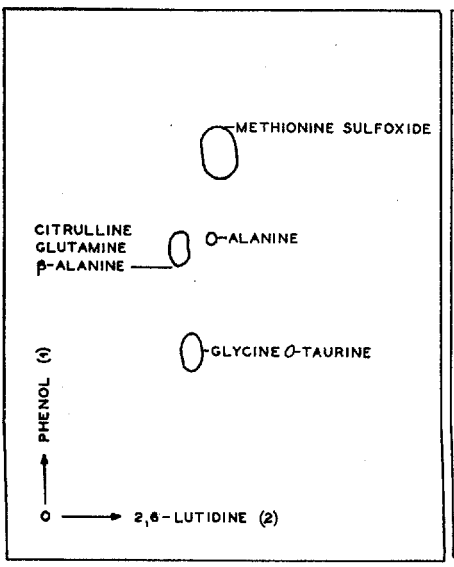

FIGURE 1

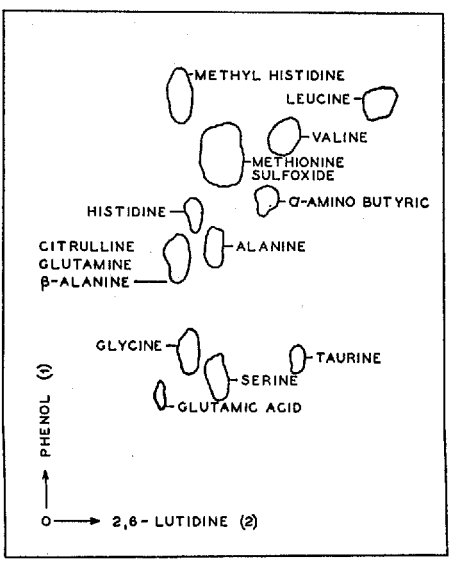

FIGURE 2

represents the chromatogram from a single particular urine sample from a given individual. In each case, however, several chromatograms of samples taken from each subject at different times were available for comparison, and the patterns as pictured are characteristic respectively of the 10 individuals.

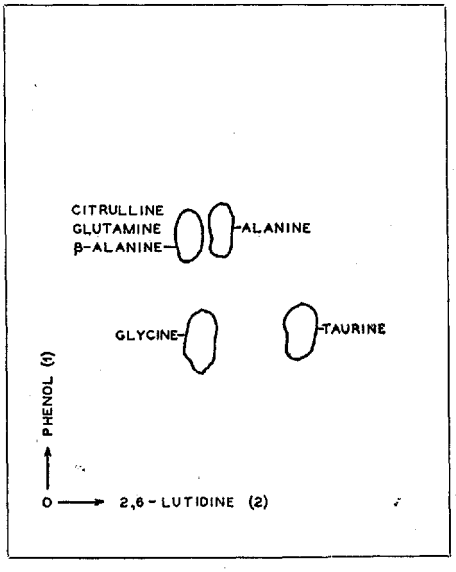

FIGURE 3

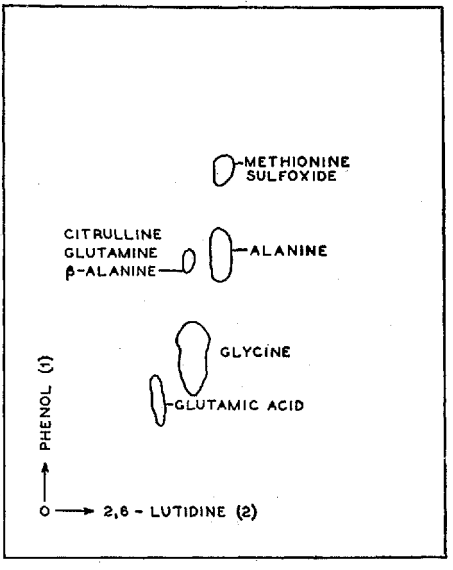

FIGURE 4

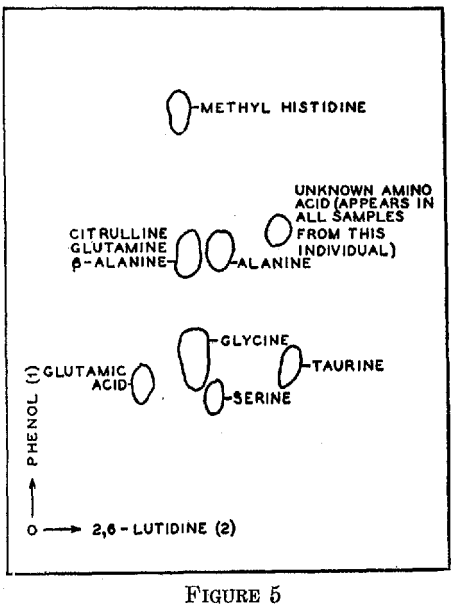

FIGURE 5

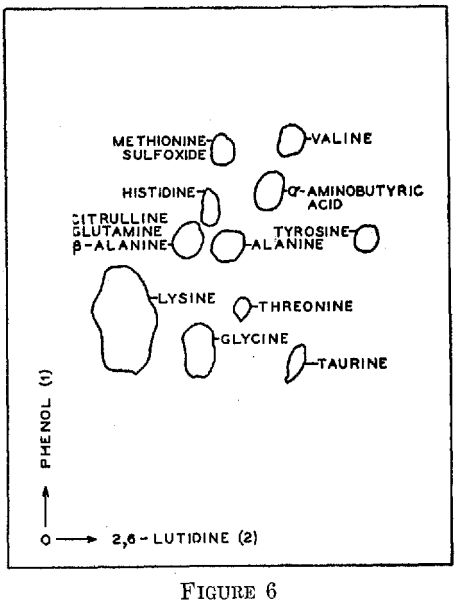

FIGURE 6

The chromatograms were prepared using phenol as the first solvent and 2,6–lutidine as the second solvent. Color development was based upon the use of 0.2% ninhydrin in water-saturated butanol. This reagent was sprayed on the sheets and they were then heated seven minutes at 90°C.

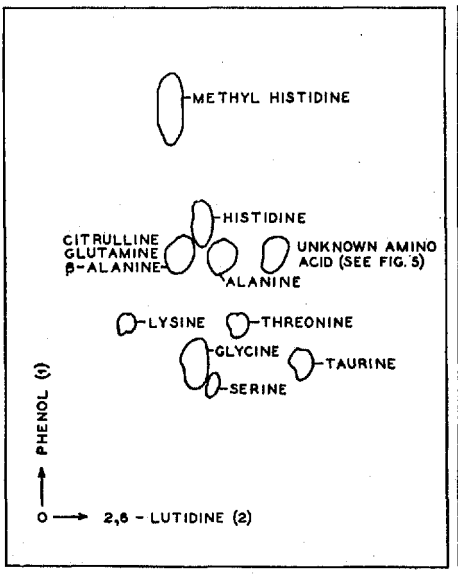

FIGURE 7

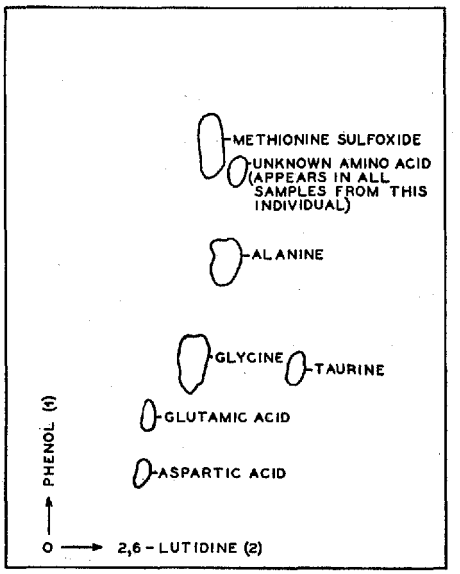

FIGURE 8

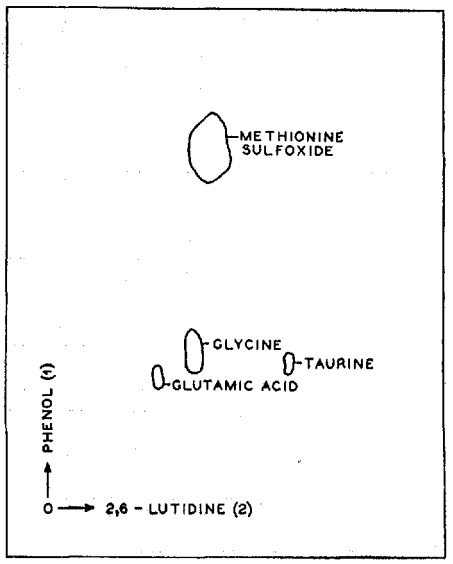

FIGURE 9

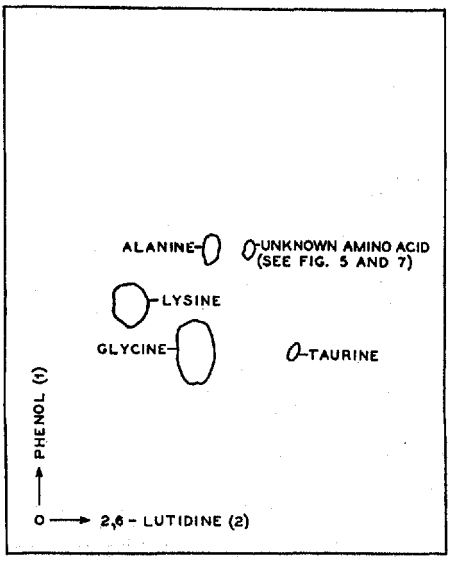

FIGURE 10

It may be noted that on Figures 5, 7 and 10 a spot appears which corresponds to an unidentified amino acid. This substance appears consistently in the urine of the three individuals indicated and also in samples from other individuals not included in this particular study. This unknown substance has an Rf value of .55 in phenol and .40 in 2,6–lutidine.

It will also be noted that one spot is designated as "citrulline-glutamine-β-alanine." All three of these substances have been detected in urine samples. They are not separated using these two solvent mixtures and hence are not differentiated.

Urinary amino acid data.—In Table I are presented data on the urinary amino acid excretions of 52 individuals derived from chromatographing a total of 178 urine samples—a minimum of 3 samples from each individual. Included in the group are 35 males and 17 females; 12 mental patients and 40 well individuals; 7 children and 45 adults.

TABLE I

Urinary Amino Acid Excretion Patterns

(amino acid concentrations expressed as μg./mg. creatinine)

No.‖	Amino Acid	Subject	1M*	2	3M	4M	5	6	7	8
		Sex	M	F	M	M	F	F	F	F
		No. Samples	3	3	3	3	3	3	3	3
4	glycine		+‡	37	+	+	32	43	42	54
6	alanine		+	4	+	+	20	25	26	21
7	citrulline		+	8	+	+	20	16	18	22
3	serine		+	19	+	+	26	24	25	19
5	taurine		+	15	+	+	18	24	18	19
10	methionine sulfoxide**		20	50	—	10	10	50	2	10
8	valine		10	10	10	8	—	5	5	10
2	glutamic acid		+	—	+	+	—	—	—	—
13	tyrosine		15	10	20	15	30	5	5	5
9	leucine		10	5	15	10	10	5	5	5
11	lysine		50	5	—	—	5	10	—	5
20	cystine		20	—	15	20	—	—	—	—
16	unidentified		10	10	—	—	20	—	5	—
17	methyl histidine		—§	5	5	10	15	10	5	5
12	threonine		—	5	15	—	15	5	5	5
18	proline		—	—	—	—	—	—	—	—
19	α–amino butyric acid		—	—	—	—	—	—	—	—
28	methionine sulfone		10	—	10	15	—	—	—	—
1	aspartic acid		—	—	—	—	—	—	—	—

*M denotes mental patient.

†C denotes child.

‡This designation (+) is used to indicate that the amino acid was found but that quantitative information is not recorded here. With regard to the mental patients and the corresponding controls, quantitative data are reported in another portion of this bulletin.

§This designation (—) indicates that the amino acid was not found by the procedure used.

‖For the significance of this see Figure 1, p. 75.

**See footnote p. 75.

TABLE I—(Continued)

Urinary Amino Acid Excretion Patterns

Spot No.	9M M 3	10M M 3	11M M 3	12C† M 3	13 M 4	14 F 3	15M M 3	16M M 6	17M M 3	18 M 3	19 M 4	20 M 4	21C F 3	22 F 3	23 F 3
4	+	+	+	54	+	49	+	+	+	+	+	+	410	75	95
6	+	+	+	20	+	14	+	+	+	+	+	+	110	29	29
7	+	+	+	44	+	17	+	+	+	+	+	+	23	29	49
3	+	+	+	29	+	17	+	+	+	+	+	+	58	38	45
5	+	+	+	26	+	21	+	+	+	+	+	+	73	48	28
21	50	20	5	80	—	10	20	5	10	10	15	10	40	10	5
8	4	20	20	15	5	5	4	16	5	—	5	10	50	—	—
2	—	+	+	7	—	+	—	+	—	+	+	+	69	+	—
13	5	5	15	—	10	5	15	10	10	10	10	15	—	5	5
9	2	8	30	10	—	—	8	7	5	—	5	10	—	5	5
11	5	6	185	15	5	—	50	—	70	8	—	—	—	—	50
20	10	20	10	20	—	—	5	10	5	—	—	—	—	—	—
16	—	—	—	10	—	—	—	—	—	—	—	—	—	—	10
17	—	—	—	—	10	5	—	—	—	10	—	10	—	—	—
12	—	—	—	5	—	10	—	—	—	5	5	—	15	10	5
18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
19	15	—	—	—	5	—	—	—	—	—	—	—	20	—	—
1	—	—	—	—	—	—	—	—	—	—	—	—	15	—	—

TABLE I—(Continued)

Urinary Amino Acid Excretion Patterns

Spot No.	24 M 7	25 M 4	26 F 3	27 F 3	28M M 3	29M M 3	30 M 4	31 M 4	32C M 4	33M M 3	34 M 6	35 M 4	36 F 3	37 M 3	38 M 3
4	+	+	42	91	+	+	+	+	110	+	+	+	31	+	+
6	+	+	12	26	+	+	+	+	50	+	+	+	10	+	+
7	+	+	15	29	+	+	+	+	—	+	+	—	10	+	+
3	+	+	21	32	+	+	+	—	—	+	+	—	20	+	+
5	+	+	7	33	+	+	+	+	130	+	—	+	26	+	+
21	5	15	5	5	30	10	—	—	70	—	2	—	—	—	—
8	—	5	—	5	25	12	10	5	30	—	2	5	5	—	—
2	—	+	—	—	+	+	+	+	130	+	—	+	—	—	+
13	5	—	5	—	—	—	—	15	—	—	—	—	—	—	50
9	—	5	5	5	—	—	—	10	20	—	—	8	—	—	—
11	8	—	—	—	—	—	3	—	50	—	4	—	—	2	—
20	10	—	—	—	10	20	—	—	—	20	—	—	—	—	—
16	15	—	5	5	—	—	—	—	—	—	3	—	15	10	—
17	—	—	—	—	—	—	10	10	—	10	—	20	—	—	—
12	—	—	—	—	—	—	—	—	—	—	—	5	—	—	—
18	—	30	—	—	—	—	—	—	30	—	—	—	—	—	—
19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

TABLE I—(Continued)

Urinary Amino Acid Excretion Patterns

Spot No.	39C F 3	40 F 3	41 F 3	42 M 3	43 M 3	44 M 3	45C F 3	46 M 5	47 M 4	48 M 4	49C F 3	50 M 4	51C M 3	52 M 3	% of Subjects Excreting Amino Acid
4	130	56	47	+	+	+	500	+	+	+	260	+	56	+	100
6	77	19	8	+	+	+	96	+	+	+	16	+	—	+	98
7	99	19	9	+	+	+	—	+	+	+	—	—	—	+	88
3	47	23	20	—	+	+	—	+	—	+	—	—	—	—	81
5	—	15	22	—	+	+	—	+	+	—	—	+	—	—	81
21	60	—	—	—	8	5	60	8	—	—	20	—	5	—	69
8	15	—	—	—	—	—	15	—	—	—	—	—	15	—	62
2	68	—	—	+	—	—	120	—	—	+	—	+	7	—	56
13	—	5	—	—	—	—	—	—	—	—	—	—	—	—	50
9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	46
11	—	—	—	—	—	—	10	—	—	—	30	—	—	—	40
20	—	—	—	5	—	—	—	—	—	—	—	—	—	—	29
16	—	—	10	10	—	10	—	—	—	—	—	—	—	—	29
17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	29
12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	27
18	—	—	—	—	—	—	—	—	—	—	30	—	—	—	6
19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

A major part of the information was derived from anaylses of a preliminary nature performed before the methods were refined to the extent that they were for succeeding studies. The preliminary studies of the mental patients and of the children were subsequently expanded into more thorough and extensive investigations which are reported elsewhere in this bulletin.

It will be noted that the amino acids, etc., in Table I are listed in the order of the frequency of their urinary occurrence. They range from glycine and alanine which are excreted by all and all but one of the individuals respectively, down to proline, α-amino butyric acid, and methionine sulfone which were found to be excreted by three individuals and to aspartic acid which was found by the procedure employed to be excreted by only one. The range of concentration for glycine in the recorded value is from 31 to 500 μg. per mg. of creatine. For alanine it was 4 to 110 μg. per mg. of creatinine. For citrulline on the same basis (excluding, of course, those individuals who were not found to excrete measurable amounts) the range was from 8 to 99. For serine the corresponding range was not so large—17 to 58. For taurine it was 7 to 130. The wide variations which exist emphasize the fact that methods of determination need not be more than crudely quantitative in order to establish the existence of distinctive patterns of excretion.

Salivary amino acid secretion patterns.—Table II gives data regarding salivary amino acid secretion patterns for a number of individuals. The saliva samples were collected immediately after the subjects arose, and no stimulus was used to induce saliva flow. The saliva was chromatographed (one dimension only) in amounts of 25 to 200 microliters using the phenol-sodium citrate-sodium phosphate solution previously described (p. 25). The table is constructed in a manner similar to that of Table I, except that the quantitative values are expressed as micrograms per milliliter of saliva.

TABLE II

Salivary Secretion of Amino Acids

Subject	Sex	No. Samples	Aspartic Acid mg./ml.	Glutamic Acid mg./ml.	Serine mg./ml.	Glycine mg./ml.	Alanine mg./ml.	Lysine mg./ml.	Unknown Amino Acid Rf–.65 in phenol
A	(f)	21	.0017	.013	.012	.014	.013	.015	.017
B	(f)	24	.0013	.013	.005	.010	.005	.0015	.017
C	(m)	2	-----	.010	-----	.014	.012	-----	.035
D	(f)	1	-----	-----	-----	-----	-----	-----	.050
E	(m)	2	-----	.020	-----	.036	.029	-----	.045
F	(m)	1	-----	.005	-----	.005	.015	-----	.005
G	(m)	1	-----	.0075	-----	.005	.012	-----	.015
H	(m)	3	.0033	.007	.0033	.008	.007	.010	.016
I	(m)	1	-----	.005	.005	.005	.010	.005	.020
Average			.0015	.012	.0066	.014	.012	.0077	.024

The existence of consistent individual patterns of secretion is quite apparent. One individual secreted no detectable amount of any of the known amino acids, four individuals secreted only 3 of the 6 known amino acids listed, one secreted 5, while the remaining three secreted all of them, but in varying amounts. It is of especial interest that the individual A whose saliva contains ten times as much lysine as individual B is the same individual whose urinary lysine is unusually high (p. 154). This indicated that the high urinary lysine is not due merely to a kidney difference, but has a broader metabolic significance.

Summary

Data are presented showing the existence of highly individual amino acid excretion patterns by 10 individuals using two dimensional chromatography. Tabulated data are also given for the

urinary excretion of amino acids and related compounds by 52 individuals (at least 3 samples from each). The amino acid content of salivas from 9 different people are also tabulated. One amino acid of unknown nature is consistently present in relatively high concentration. One individual who excretes relatively large amounts of urinary lysine was found to have a high content in the saliva also.

XVIII. Individual Urinary Excretion Patterns of Young Children

by

HELEN KIRBY BERRY AND LOUISE CAIN

Urine samples from young children were analyzed following procedures developed for studying metabolic excretion patterns, in order to gain background information as to the existence of patterns in early life and how they compare with those of adults. During the early months of life children receive a relatively standard diet in which milk is the main item. If differences in diet are in a considerable measure responsible for differences in patterns, then one would not expect to find differences in patterns to be prominent in early ages.

Experimental

Subjects.—Ten male and 11 female children ranging in age from 6 weeks to 7 years were employed in this study. The ages of the various subjects are shown in Table I. The boys, H2, H3, and H4, and the girl H1 were siblings; of the girls, B1, B2, and B3 were sisters; the other subjects were unrelated. The diets of the children were not controlled or modified in any way.

Urine samples.—Three urine samples (morning samples wherever feasible) were collected on consecutive days. The effect of varying urine concentration was minimized by expressing all results using creatinine as a base (p. 152).

Analytical methods.—Creatinine was determined by a standard colorimetric procedure (1). All other constituents were determined by paper chromatographic methods. Amino acids, except histidine, were determined on one-dimensional paper chromatograms using phenol as solvent and ninhydrin as the color developing reagent (p. 71). In some instances, where there were interferences on the one-dimensional chromatograms, two-dimensional chromatograms (p. 72) were employed. Urea was determined on paper chromatograms resolved with phenol and developed for color with sodium hypochlorite (p. 29). Phosphate was determined on chromatograms resolved in butanol-acetic acid solvent, and developed for color with ferric chloride solution (p. 28). Another chromatogram resolved with the butanol-acetic acid solvent was sprayed

with bromcresol green reagent (p. 29) for the location of acidic areas due to such constituents as citric acid, lactic acid and hippuric acid; and of a basic area which can be interpreted as a measure of total "weak acids" present (p. 30). Still another chromatogram resolved in the butanol-acetic acid solvent was developed for color with the *p*-dimethylaminobenzaldehyde reagent (p. 30). A number of characteristic spots whose identities have not been determined are developed by this reagent. Histidine and an additional group of unidentified constituents were determined on chromatograms resolved with the butanol-ethanol-hydrochloric acid solvent (p. 77) and the butanol-acetic acid solvent (p. 25) respectively, and were developed for color with the diazotized sulfanilic acid reagent (p. 30). Another unknown urinary constituent of common occurrence was determined on a chromatogram resolved in the isobutyric acid-acetic acid solvent (p. 26) and developed for color with the bromine reagent (p. 30).

Results

The averages of determinations on three urine samples from each of the subjects are given in Table I. A blank in the table indicates that the constituent in question was not determined. A dash in the table indicates that determinations were performed but nothing was detected. Values for creatinine are expressed in mg./ml. of urine. Values for urea and the amino acids are expressed in mg. of constituent per mg. of creatinine. The other constituents are either unidentified, or some doubt exists as to their identity, and the quantitative values for these substances are expressed in terms of the area of the spot developed from a quantity of urine equivalent to 40 micrograms of creatinine; however, in the case of those substances developed for color with diazotized sulfanilic acid, the areas reported were developed from a quantity of urine equivalent to 100 micrograms of creatinine. Most of the data, it will be seen, are expressed relative to creatinine. A justification for this procedure will be found elsewhere in this bulletin (p. 152), though actually the justification has not been shown to exist in the case of very young children. Since previous workers (2,3) have indicated that the excretion of creatinine is a direct function of body weight, or, more particularly of muscle tissue weight, the results expressed in terms of creatinine provide a rough means of comparing the excretion of different children in terms of mg. of constituent per unit body weight. It should be borne in mind that the areas recorded as quantitative measures of certain of the con-

TABLE I

Urinary Excretion Patterns in Young Children

Subjects	Age in months	Creatinine mg./ml.	Urea mg./ml.	PO_4 Area (in.²) corres. 40 μg. Cr.	Histidine mg./ml.	Glutamic mg./ml.	Serine mg./ml.
Females							
A	5	0.09	41	0.31	.....	.13	.....
B1	11	0.22	36	0.31	0.22	.060	.....
C	18	0.52	37	0.29	0.15	.12	.11
D	19	0.44	52	0.26	0.29	.010	.015
E	29	0.45	40	0.45	0.17	.068	.047
F	32	0.70	45	0.22	0.30	.037	.065
G	36	0.62	31	0.29	0.36	.069	.058
B2	36	0.36	30	0.22	0.39	.023	.....
H1	48	0.78	17	0.09	0.08	.044	.017
I	48	0.72	50	0.17	0.41		
B3	54	0.30	50	0.11	0.18	.059	.071
Males							
J	1½	0.13	33	0.26	.....	.13	.....
K	11	0.34	66	0.42	0.07	.030	.021
L	19	0.99	50	0.45	0.19	.007	.029
M	19	0.39	30	0.29	0.36	.22	.13
H2	20	1.27	16	0.23	0.25	.044	.070
N	21	0.54	45	0.26	0.06	.021	.....
O	37	0.91	33	0.20	0.09	.007	.007
H3	42	0.92	17	0.08	0.23	.017	.11
H4	69	0.88	32	0.22	0.13	.020	.050
P	84	1.28	23	0.15	0.35		
Average:							
10 boys		0.76	35	0.26	0.19	0.055	0.046
11 girls		0.51	39	0.24	0.26	0.054	0.042

Subject	Glycine mg./ml.	Taurine mg./ml.	Alanine mg./ml.	Citrulline mg./mg.Cr.	Methionine Sulfoxide* mg./mg.Cr.
Females					
A	.047	.....	.....	.....	.11
B1	.62	.....	.074	.013	.18
C	.50	.....	.096	.031	.046
D	.26	.006	.016	.....	.018
E	.13	.....	.077	.099	.061
F	.091	.11	.043	.027	.052
G	.41	.073	.11	.023	.041
B2	.069	.....	.047	.023	.019
H1	.072	.027	.004	.013	.....
I				.....	.....
B3	.18	.024	.....	.....	.039
Males					
J	.11	.13	.050	.....	.071
K	.023	.....	.....	.....	.032
L	.054	.026	.020	.044	.079
M	.22	.....	.10	.025	.065
H2	.13	.026	.050	.063	.016
N	.005	.038	.....	.....	.013
O	.056	.....	.006	.011	.004
H3	.17	.....	.067	.067	.....
H4	.10	.012	.046	.044	.....
P				.....	.....
Average:					
10 boys	0.096	0.026	0.038	0.028	0.031
11 girls	0.26	0.027	0.052	0.025	0.052

*See footnote p. 75.

stituents listed in Table I are probably not linear functions of concentration (p. 50), and while the existence of differences may be validly inferred, the magnitude of these differences is not accurately indicated.

Discussion

The data of Table I strongly indicate the existence of characteristic excretion patterns for the children studied. The spread between the highest and lowest individual values recorded for the eleven identified constituents, beginning with histidine, averages about 26 fold and in the case of no item is the spread less than 7 to 1. This statement tends to minimize the differences, because for 8 of the 11 items, several individuals showed no detectable quantity. Furthermore the differences with respect to the unidentified constituents appear to be greater than for the identified ones.

TABLE I—(Continued)

Color developed with bromcresol green reagent

Subject	(citric) yellow Rf .24	(lactic) yellow Rf .79	(hippuric) yellow Rf .33	"basic cations" blue Rf .33	Color developed with Bromine Reagent Rf .40
	(Area (in.^{2}) corresponding to 40 γ creatinine)				
Females					
A	-----	-----	-----	-----	.03
B1	-----	-----	.31	.95	-----
C	.50	-----	.32	.26	-----
D	.07	.16	.02	.17	.10
E	.52	-----	-----	.38	-----
F	.63	.16	.07	-----	.18
G	.50	-----	.17	.15	.10
B2	.16	-----	.06	.27	.11
H1	.25	.14	.07	.17	.16
I	.13	.08	.06	-----	.10
B3	-----	-----	.06	.07	.08
Males					
J	-----	.65	.13	1.20	.06
K	.15	-----	-----	.82	-----
L	.55	-----	.25	-----	-----
M	-----	-----	.30	.95	.13
H2	.36	.26	.28	-----	.13
N	-----	.09	-----	.44	.18
O	.26	-----	-----	.10	-----
H3	.33	.23	.21	-----	.13
H4	.45	.16	.16	-----	.11
P	.08	.07	-----	-----	.09
Average:					
10 boys	0.22	0.15	0.13	0.35	0.12
11 girls	0.28	0.05	0.11	0.24	0.10

TABLE I—(Continued)

Color developed with diazotized sulfanilic acid

Subject	pink Rf .20	pink Rf .23	br. pk. Rf .26	orange Rf .33	orange Rf .52	yellow Rf .59	pink Rf .60	orange Rf .80	pink Rf .92
	(Area (in.²) corresponding to 100 γ creatinine)								
Females									
A	-----	-----	-----	-----	-----	-----	-----	-----	-----
B1	-----	-----	-----	-----	-----	-----	.007	.17	.18
C	-----	.19	-----	-----	-----	-----	.14	.17	.05
D	-----	.13	-----	-----	-----	-----	.03	.18	.08
E	-----	.05	.28	-----	-----	-----	.22	.18	.20
F	-----	.17	-----	-----	-----	-----	-----	.06	.11
G	-----	.14	-----	-----	-----	-----	.08	.06	.11
B2	-----	.13	-----	-----	-----	-----	-----	.10	.04
H1	.08	.13	-----	-----	-----	-----	-----	-----	.04
I	-----	.35	-----	-----	-----	-----	.05	-----	.05
B3	-----	.03	-----	-----	-----	-----	-----	-----	-----
Males									
J	-----	.30	-----	-----	-----	-----	.13	.04	.09
K	-----	.31	-----	-----	-----	.08	.09	.23	.12
L	-----	.29	-----	-----	-----	-----	.25	.23	.18
M	-----	.18	.13	.13	1.02	-----	.04	.40	.20
H2	-----	.13	-----	-----	-----	-----	.10	.14	.14
N	-----	-----	-----	-----	-----	-----	.02	-----	.08
O	-----	.20	-----	-----	-----	-----	.12	.17	.11
H3	.08	.08	-----	-----	-----	-----	-----	.24	.09
H4	.30	.27	.30	-----	-----	-----	-----	.17	.09
P	-----	.16	-----	-----	-----	-----	-----	-----	.12
Average:									
10 boys	-----	0.19	-----	-----	-----	-----	0.07	0.16	0.12
11 girls	-----	0.13	-----	-----	-----	-----	0.05	0.09	0.09

TABLE I—(Continued)

Color developed with p-dimethylamino benzaldehyde reagent

Subject	yellow Rf .33	purple Rf .35	brown Rf .40	grey Rf .77	pink Rf .88	purple Rf .92	(allantoin) yellow Rf .20
	(Area (in.²) corresponding to 40 γ creatinine)						
Females							
A	-----	-----	-----	-----	-----	-----	.33
B1	-----	-----	-----	-----	-----	.24	-----
C	-----	-----	.16	.24	-----	-----	-----
D	-----	-----	-----	.18	-----	.18	-----
E	-----	-----	.21	.10	-----	.26	-----
F	-----	.15	.18	-----	-----	.17	-----
G	-----	-----	-----	-----	-----	-----	-----
B2	-----	-----	-----	-----	-----	-----	-----
H1	-----	.16	.22	-----	-----	-----	-----
I	-----	-----	-----	-----	-----	-----	-----
B3	-----	-----	-----	-----	-----	-----	-----
Males							
J	-----	.14	-----	-----	.18	.14	.35
K	-----	-----	-----	-----	-----	.19	-----
L	-----	-----	-----	.22	-----	-----	-----
M	-----	-----	-----	-----	-----	.13	-----
H2	-----	-----	-----	-----	-----	-----	-----
N	-----	-----	-----	-----	.17	.14	-----
O	-----	-----	.24	-----	-----	-----	-----
H3	-----	.19	.30	-----	-----	-----	-----
H4	-----	.15	.31	-----	-----	-----	-----
P	-----	-----	-----	-----	-----	-----	-----

Inspection and statistical analysis of the results indicate the presence of some sex differences. For example, the average excretion of glycine for the girls is nearly three times that for the boys. A difference in the same direction has also been observed in the case of adults (p. 154). In general, the average values of the constituents appear remarkably uniform for the sexes. There also appear to be a few age differences. Only the two youngest children were found, for example, to excrete detectable amounts of allantoin. Creatinine excretion, as might be expected, is low in the youngest children.

Since initial inspection of the data appeared to indicate that the three brothers included in the study (H2, H3, and H4, ages 5½, 3½ and 1½ respectively) showed patterns which resembled each other strikingly, we applied statistical analysis to reveal objectively the extent of similarity of all the siblings involved in the study. The "coefficients of similarity"* between each of the tabulated values for each of these brothers and the corresponding values for the other brothers and for five other boys in about the same age group were calculated. The same type comparison was made for the three sisters (B1, B2, and B3, ages 1, 3 and 4½ respectively) and five girls of about the same age group. The girl, H1, age 4, was compared to her three brothers and to the three sisters from another family B1, B2, and B3. For this purpose the same 11 consecutive items beginning with histidine in Table I were used. Where a substance was not detected in a sample, it was taken, for purposes of calculation, to be present in an amount equal to one-half the lowest amount found in any of the samples. The results of these calculations are shown in Table II.

These calculations definitely confirm the observation as to the pronounced similarities of the brothers' patterns and strongly suggest that these patterns of excretion are inherited and are relatively stable, since the three brothers exhibited similarity in patterns regardless of their ages. On the other hand, the three sisters showed no greater average similarity to each other than to the five unrelated girls with whom they were compared. The three brothers' sister, H1, showed only a slightly greater average similarity to her brothers than to the three sisters; this slight difference is probably not significant. Since H1 and her three brothers presumably have access to the same food the comparative dissimilarity between the girl and her brothers is in line with the idea

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* "coefficient of similarity" $= \frac{\text{lower value}}{\text{average value}} \times 100$

TABLE II

		Average Coefficient of Similarity for 11 Items	Average of Average Coeff. of Similarity
1. a.	Between brothers		
	H4 and H3	80	
	H4 and H2	73	76
	H3 and H2	76	
b.	Between male non-siblings		
	H4 and K	46	
	H4 and L	65	
	H4 and M	46	
	H4 and N	40	
	H4 and O	53	
	H3 and K	44	
	H3 and L	54	
	H3 and M	58	48
	H3 and N	28	
	H3 and O	50	
	H2 and K	41	
	H2 and L	64	
	H2 and M	53	
	H2 and N	38	
	H2 and O	39	
2. a.	Between sisters		
	B1 and B2	62	
	B1 and B3	56	57
	B2 and B3	52	
b.	Between female non-siblings		
	B1 and C	59	
	B1 and D	49	
	B1 and F	44	
	B1 and G	59	
	B1 and H1	43	
	B2 and C	56	57
	B2 and D	60	
	B2 and F	63	
	B2 and G	64	
	B2 and H1	58	
	B3 and C	53	
	B3 and D	54	
	B3 and F	57	
	B3 and G	61	
	B3 and H1	59	
3. a.	Between the sister and her three brothers		
	H1 and H3	53	
	H1 and H2	57	59
	H1 and H4	66	
b.	Between the same girl and three sisters not her siblings		
	H1 and B1	43	
	H1 and B2	58	53
	H1 and B3	59	

that the differences are of an internal nature. The existence of strong similarity between certain siblings and lack of similarity between others, as in the sisters above, is in line with what would be expected on a hereditary basis.

It may be noted that of the non-sibling boys, L's pattern is most like that of the three brothers and N's pattern is least like them; the average coefficient of similarity for L is 61, while for N the corresponding value is 35. Whether these similarities and differences in patterns denote similarities and differences in patterns of mental abilities or personality traits is unknown and can only be determined by extended interdisciplinary research.

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XIX. A Further Study of Urinary Excretion Patterns in Relation to Diet

by

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All of the studies presented in this bulletin both on animals and human beings indicate that excretion patterns are determined in large part by factors other than diet. It is well known, however, that unusual urinary constituents do appear as the result of eating particular foods (of which asparagus is an outstanding example) and the question of diet seemed worth investigating again from the standpoint of individual excretion patterns. For one thing it was hoped that additional light might be thrown on the problem of whether the observed individual patterns are caused in part by differences in the self selection of foods by the individuals concerned. In an earlier experiment reported from this laboratory (1) several individuals were placed on identical diets but only four urinary amino acids were determined (microbiologically) and found to be essentially unchanged by changes in diet. The present experiment was similar in principle to the former one; many more items were included but fewer individuals participated.

Experimental

Preliminary studies failed to show that the use of special foods, including asparagus, caused the appearance of any unusual urinary constituent as determined by paper chromatography. It is known that asparagus consumption causes the excretion of methyl mercaptan which has a characteristic odor (2). Due presumably to the minute amounts present and to its volatility this substance does not show up on chromatograms. No quantitative information is available as to the amounts of methyl mercaptan which may be present in urine, but because the sense of smell for compounds of this type is extremely sensitive, it is probable that the amounts are extremely minute (3).

For a more systematic study three young healthy males were chosen because in preliminary experiments they apparently exhibited noticeable differences in their urinary excretion patterns. During the experimental period of five consecutive days, these individuals ate together at all times, choosing a diet that did not differ greatly from the normal regimen of any of them. No attempt was

made to eat the same foods on each day of the five day period; rather the diet was varied as much as possible, keeping within the realm of "common" foods. Urine samples were collected upon rising for three mornings prior to the experimental period, for the five days of the experimental period, and for the three mornings following. The urine samples were frozen until ready for analysis.

No attempt was made to control the amount of exercise of each person, but as the subjects are in similar occupations and undergo approximately the same amount of exercise, no error should be anticipated from this source. None of the subjects engaged in an unusual amount of exercise during the experimental period.

The analytical procedures used were similar to those used in previous studies of this nature performed in this laboratory. Creatinine was determined by the picrate method (4) and was used as a basis for comparing the results of other analyses in order to overcome the dilution factor. The amino acids were determined on two-dimensional paper chromatograms according to the techniques reported by Berry and Cain (5) with the exception of histidine which was done by the method reported elsewhere in this bulletin (p. 77). The compounds which form colored products with diazotized sulfanilic acid were detected by chromatographing on paper an aliquot of urine containing 100 micrograms of creatinine, resolving it in the butanol-acetic acid-water mixture (p. 25), and developing the resulting sheet by spraying with diazotized sulfanilic acid (p. 30). The substances reacting with bromcresol green (p. 29) were chromatographed by resolving in the butanol-acetic acid-water mixture an aliquot of urine containing 60 micrograms of creatinine. Ferric chloride chromogens (p. 28) were likewise detected using an aliquot of urine containing 40 micrograms of creatinine. Chromatograms were also prepared by resolving aliquots of urine containing 40 micrograms of creatinine in the butanol-acetic acid-water mixture and developing respectively with ammoniacal silver nitrate (p. 27), 2,6-dichloroquinonechloroimide (p. 33), p-dimethylaminobenzaldehyde (p. 30), or bromine reagent (p. 30). The substances developed by these last reagents are for the most part either the same as detected by other methods of analysis or of unknown composition with no accurate means of quantitation. Visual comparison of the size and intensity of the urea and the uric acid spots as well as those of other chromogens indicated that there was no significant difference between samples when considered from the point of view of either the individual or the diet; hence, no further attempts were made to determine these substances quantita-

tively. Phosphate was determined by the colorimetric method of Fiske and Subbarow (6).

Results

The results of these determinations are shown in Table I, most of the data being expressed using creatinine as a basis. Where no absolute standard was used for reference and the spots on the chromatograms were of sufficient resolution for the area to be accurately measured with a polar planimeter, the results are expressed in square inches. When the spots could only be compared visually for intensity, the results are expressed as —, +, ++, etc.

TABLE I

	Creatinine (mg./ml.)			Histidine hydrochloride (mg./mg. Cr.)		
Day	Subj. A	Subj. B	Subj. C	Subj. A	Subj. B	Subj. C
C1	0.96	2.70	2.14	0.10	0.07	0.05
C2	1.68	1.74	1.92	0.06	0.17	0.16
C3	1.02	1.12	2.64	0.10	0.20	0.15
E1	1.12	1.06	1.80	0.09	0.14	0.17
E2	1.26	3.46	2.46	0.10	0.10	0.16
E3	1.04	1.38	1.76	0.10	0.22	0.17
E4	1.08	1.94	1.44	0.09	0.15	0.10
E5	1.14	1.86	1.42	0.07	0.19	0.21
C4	0.80	1.32	2.62	0.11	0.15	0.17
C5	1.52	0.92	2.98	0.09	0.19	0.13
C6	0.82	2.20	2.58	0.10	0.14	0.14

	Glycine (mg./mg. Cr.)			Alanine (mg./mg. Cr.)		
Day	Subj. A	Subj. B	Subj. C	Subj. A	Subj. B	Subj. C
C1	0.07	0.07	0.03	0.02	0.01	0.02
C2	0.02	0.05	0.04	0.02	0.01	0.02
C3	0.03	0.12	0.04	0.02	0.04	0.01
E1	0.04	0.03	0.06	0.01	0.03	0.02
E2	0.02	0.03	0.04	0.01	0.01	0.01
E3	0.015	0.06	0.07	0.01	0.02	0.02
E4	0.04	0.03	0.04	0.02	0.03	0.02
E5	0.04	0.05	0.07	0.005	0.02	0.02
C4	0.05	0.06	0.06	0.02	0.02	0.02
C5	0.05	0.04	0.04	0.01	0.04	0.01
C6	0.05	0.05	0.03	0.02	0.01	0.01

	Serine (mg./mg. Cr.)			Citrulline (mg./mg. Cr.)		
Day	Subj. A	Subj. B	Subj. C	Subj. A	Subj. B	Subj. C
C1	0.03	0.02	0.03	0.06	0.09	0.07
C2	0.02	0.03	0.04	0.06	0.06	0.10
C3	0.03	0.04	0.03	0.04	0.12	0.05
E1	0.03	0.03	0.03	0.03	0.03	0.11
E2	0.02	0.02	0.02	0.06	0.06	0.10
E3	0.01	0.04	0.02	0.04	0.07	0.09
E4	0.02	0.03	0.03	0.07	0.07	0.05
E5	0.03	0.02	0.05	0.06	0.11	0.05
C4	0.05	0.02	0.03	0.12	0.05	0.05
C5	0.03	0.02	0.02	0.05	0.08	0.07
C6	0.05	0.03	0.03	0.07	0.06	0.06

TABLE I—(Continued)

Day	Taurine (mg./mg. Cr.) Subj. A	Subj. B	Subj. C	Methionine Sulfoxide* (mg./mg. Cr.) Subj. A	Subj. B	Subj. C
C1	0.00	0.07	0.07	0.07	0.06	0.03
C2	0.10	0.11	0.06	0.04	0.04	0.04
C3	0.00	0.11	0.06	0.12	0.11	0.01
E1	0.09	0.14	0.11	0.06	0.09	0.03
E2	0.08	0.07	0.10	0.06	0.06	0.10
E3	0.02	0.11	0.11	0.02	0.07	0.06
E4	0.11	0.08	0.10	0.10	0.05	0.04
E5	0.09	0.13	0.25	0.05	0.06	0.04
C4	0.19	0.11	0.08	0.05	0.08	0.02
C5	0.05	0.08	0.07	0.08	0.11	0.02
C6	0.07	0.05	0.10	0.08	0.03	0.10

Day	Valine (mg./ml.) Subj. A	Subj. B	Subj. C	Leucine (mg./ml.) Subj. A	Subj. B	Subj. C
C1	0.03	0.02	-----	0.09	-----	-----
C2	0.02	0.03	0.007	0.03	-----	0.03
C3	0.01	-----	-----	-----	-----	-----
E1	-----	-----	0.002	-----	-----	0.04
E2	0.02	-----	-----	0.05	-----	-----
E3	0.007	-----	-----	0.03	-----	-----
E4	-----	0.01	-----	-----	0.03	-----
E5	-----	-----	0.007	-----	-----	-----
C4	0.01	-----	-----	-----	-----	-----
C5	-----	0.01	0.01	-----	0.03	0.03
C6	-----	0.01	0.02	-----	0.03	-----

Day	"Under Citrulline" Rating/20 μl. Urine Subj. A	Subj. B	Subj. C	"Over Threonine" Rating/20 μl. Urine Subj. A	Subj. B	Subj. C
C1	++		++	++		+
C2	+		++	+		++
C3	+		+++			++
E1	+		++	+		+
E2	++		+	+		+
E3			++	+		+
E4			+	+		+
E5	+		+	+	+	+
C4			+	+	+	+
C5	+	+	++			+
C6	+		++	+		+

Day	Lysine (mg./mg. Cr.) Subj. A	Subj. B	Subj. C	Sulfanilic Acid Orange Spot (Rf .85) Rating/100 μg. Cr. Subj. A	Subj. B	Subj. C
C1	-----	0.02	0.015	+++++	++++	+
C2	-----	0.015	0.015	++++	+	+
C3	-----	-----	-----	+++	+++++	+++
E1	-----	-----	-----	++++	+++	+++
E2	-----	-----	0.01	+++	+++++	++++
E3	-----	-----	-----	++++	+++++	+++++
E4	0.02	0.015	-----	++++	++++	++++
E5	-----	-----	-----	++	+	++
C4	-----	0.01	-----	++	+	+
C5	-----	0.04	0.015	++	+	+
C6	-----	-----	0.015	++	++++	+

*See footnote p. 75.

TABLE I—(Continued)

Day	Sulfanilic Acid Purple Spot (Rf .90) Area (in.²/100μg. Cr.)			Hippuric Acid Area (in.²/60μg. Cr.)		
	Subj. A	Subj. B	Subj. C	Subj. A	Subj. B	Subj. C
C1	0.06	obscured	0.11	0.41	0.45	0.25
C2	0.00	0.07	0.15	0.63	0.33	0.35
C3	0.08	0.33	0.17	0.35	0.34	0.27
E1	0.25	0.29	0.20	0.40	0.34	0.43
E2	0.05	0.13	0.11	0.30	0.43	0.26
E3	0.12	0.15	0.22	0.32	0.44	0.40
E4	0.21	0.18	0.23	0.51	0.43	0.37
E5	0.26	0.31	0.28	0.31	0.37	0.37
C4	0.00	0.15	0.16	0.60	0.39	0.23
C5	0.16	0.46	0.15	0.25	0.29	0.26
C6	0.00	0.26	0.05	0.17	0.31	0.37

Day	Bromcresol Green Acid Spot (Rf .28) Area (in.²/60μg. Cr.)			Bromcresol Green Acid Spot (Rf .30) Area (in.²/60 μg. Cr.)		
	Subj. A	Subj. B	Subj. C	Subj. A	Subj. B	Subj. C
C1	0.15	0.13	0.17	0.00	0.07	0.00
C2	0.10	0.28	0.18	0.00	0.14	0.00
C3	0.23	0.13	0.21	0.06	0.00	0.00
E1	0.35	0.16	0.22	0.00	0.10	0.11
E2	0.33	0.10	0.19	0.00	0.07	0.04
E3	0.30	0.18	0.21	0.07	0.10	0.07
E4	0.07	0.14	0.17	0.00	0.06	0.05
E5	0.28	0.14	0.18	0.05	0.05	0.00
C4	0.19	0.27	0.25	0.00	0.10	0.07
C5	0.23	0.20	0.27	0.08	0.21	0.07
C6	0.13	0.20	0.29	0.00	0.07	0.09

Day	Phosphate (mg./mg. Cr.)		
	Subject A	Subject B	Subject C
C1	0.51	0.78	0.90
C2	0.60	0.28	0.89
C3	0.68	0.56	0.68
E1	0.56	0.98	0.77
E2	0.56	0.72	0.72
E3	0.53	0.61	0.73
E4	0.56	0.72	0.66
E5	0.69	0.98	0.89
C4	0.43	0.73	0.86
C5	0.44	0.68	0.89
C6	0.55	0.25	0.79

Two unknown substances are reported which were detected on the ninhydrin-developed amino acid chromatograms. They are "under citrulline," (ascending method) a blue spot which migrates in phenol to a position just below that of citrulline and in lutidine to the same position as citrulline, and "over threonine," a purple spot which migrates in lutidine to a position just above threonine and in phenol to the same position as threonine. In Table I the control days are indicated by the letter C, the experimental days by E. Creatinine is abbreviated Cr.

A study of the results presented in Table I does not yield as much conclusive information as might be desired, because for many of the items determined the individuals did not show, during the short period, significant differences. A number of interesting facts, however, may be noted. First, the changes in the diet which were instituted did not appear to modify the excretion of most of the substances to any material degree. Secondly, certain substances appeared at characteristic levels for each individual and did not change when the diet was modified. Histidine is a clear cut example of this, and the substances designated "under citrulline," "over threonine" and "Bromcresol Green (Rf .30)" appear to fall in the same category. Thirdly, the excretion of certain substances varies with the diet. The clearest example of this is "Sulfanilic Acid Purple (Rf .90)." When the values for this substance are plotted for the days on which collections were made, a marked parallelism is noted between the curves of the individuals during the experimental period (Fig. 1). Spot "Sulfanilic Acid Orange (Rf .85)" appears to be in the same class but the data are not so clear cut.

Not included in the tabulated material are three additional unknown substances: A sulfanilic acid red streak, Rf .22–.36, occurred in much higher quantities in the urine of subject B than in that of subjects A and C and did not seem to vary with the diet. A ferric chloride alkaline spot (Rf .34), which reflects weak acid excretion (7), consistently occurred strongly, regardless of diet, in samples from subject A, less strongly from subject B, and hardly at all from subject C. A sulfanilic acid orange spot Rf .60 occurred in all samples but seemed to vary with the diet as did the other sulfanilic acid orange spot Rf .85.

Phosphate and the bromcresol green acid spot (Rf .28) are excreted at characteristic levels, but the daily parallelism in two of the subjects indicates that the excretion of these substances may fluctuate to some extent with the diet. However, the excretion of these substances by the third subject seems not to be related to that of the others.

The majority of cases seem to fall between the two extremes. Most of the substances are excreted at levels apparently characteristic of the individuals, but the rather large daily variation of many of these substances makes it difficult to evaluate the importance of diet in their excretion. This is true of the amino acids other than histidine; in these, the accuracy of the method of determination will not allow detection of possible small changes

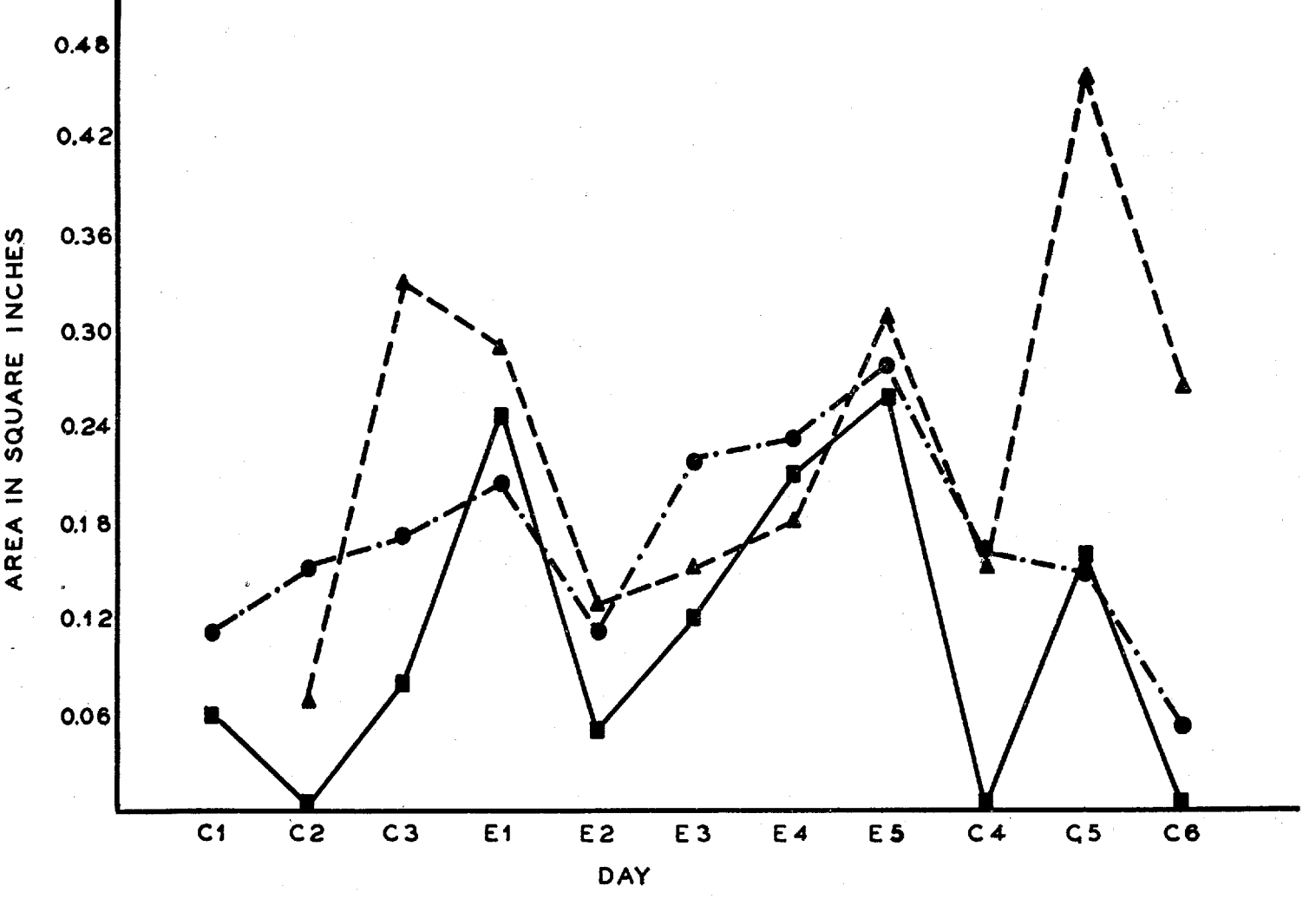

FIGURE I.

Area of Sulfanilic Acid Purple Spot (Rf. 90) vs. Day of Excretion.

caused by diet. The methods are sufficient to indicate, however, that there are no major changes due to dietary differences and that when treated statisically, the levels of excretion as a whole are characteristic of an individual.

Summary

Experiments are described in which the relationship of diet to urinary excretion patterns was studied in three individuals who were on an identical diet for a five day period. The results indicate that of the many substances which were studied, only two showed an obvious dependence upon diet. These were the sulfanilic acid orange spot (Rf .85) and the sulfanilic acid purple spot (Rf .90). Of the remainder of the substances, some may vary slightly with diet, but in no case does the diet appear to be as important in determining the excretion patterns as do other factors seemingly characteristic of the individual. Several substances, notably histidine, appeared to be excreted at levels which were quite characteristic of each of the three individuals.

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XX. Metabolic Patterns of Underweight and Overweight Individuals

by

JACK D. BROWN AND ERNEST BEERSTECHER, JR.

Recent studies by Williams, *et al.* (1–5), of individual metabolic differences have proved to be very suggestive in connection with such problems as alcoholism, mental disease, etc. With this in mind it was decided that a similar biochemical approach might be helpful in elucidating the causes for the differences between persons who are decidedly overweight and those who are underweight but who do not exhibit any other striking abnormalities. A survey of the literature reveals a considerable divergence of opinion as to the types and possible causes of obesity and leanness. The accent in the past has been on the study of obese persons as compared with so-called normals. The available information suggests the advisability of avoiding subjects who are prodigiously fat or who are of the walking-skeleton variety because in these, pathological conditions may exist which are not to be found in those who deviate less from the average. Consequently, the subjects in the present study have been selected for their tendency toward being over- or underweight. Extreme cases were not selected.

Likely male subjects, ranging in age from 20 to 31 years, were obtained through the co-operation of the University Health Service. One group of seven, henceforth designated as the overweight group, had for no apparent reason, a decided tendency to be overweight and experienced great difficulty in controlling their weight downward toward the average. Members of the underweight group, comprised of ten individuals, were definitely on the lean side and, for no apparent reason, encountered considerable difficulty in gaining weight. Prior to final selection of the above groups, prospective subjects were screened to exclude those with any previous history of diseases, such as diabetes, which might introduce factors of error into the study. Personal data of the underweight and overweight subjects studied are presented in Table I.

Urine samples were selected for use in this study because it has been shown (1–5) that a study of urinary constituents is very enlightening in observing individual metabolic differences. Urine reflects in a measure a true image of the metabolic processes which take place in the body.

No attempt was made to control the diets of the individuals being studied. As is demonstrated elsewhere in this bulletin (6), a number of urinary constituents remain relatively constant for an individual independent of his diet. That self-selection of food by the subjects should not be eliminated from consideration is further supported by other investigations (4).

TABLE 1

Personal Data of Overweight and Underweight Subjects

Subject Number	Age (years)	Height	Weight (pounds)	Difficulty in controlling wt. toward "normal"
Overweight:				
10	25	5′ 8″	215	Great
11	20	6′ 4″	262	Great
12	29	6′ 0″	255	Very great
13	28	5′ 10″	230	Very great
14	31	6′ 1″	230	Great
15	29	5′ 11½″	270	Great
16	26	5′ 9″	205	Very great
Underweight:				
20	25	6′ 0″	135	Great
21	25	6′ 0″	140	Great
22	21	6′ 0″	130	Very great
23	25	5′ 8″	122	Great
24	22	6′ 4½″	162	Very great
25	29	5′ 4″	98	Very great
26	28	5′ 11½″	135	Very great
27	27	5′ 11½″	141	Great
28	26	5′ 10″	115	Very great
29	25	5′ 11½″	128	Very great

Experimental

Three complete morning samples of urine, collected at intervals of three to five days, were obtained from each subject. The volume, pH, and specific gravity were measured for each sample, and the samples were then refrigerated under toluene.

Methods

Spectrochemical analyses of urine for phosphorus, potassium, sodium, calcium, copper, boron, magnesium, and iron were performed by the Pacific Spectro-Chemical Laboratory of Los Angeles, California, by standard techniques. Duplicate determinations revealed that the average deviation for all elements except iron and copper was 2.2%, indicating a probable error of about 3.3% of the true amount present. The probable error for iron and copper was calculated to be about 15%. In addition to the elements reported, strontium and silicon were detected in all samples. Strontium concentration appeared to vary directly with the calcium content of the urine.

The method of Bonsnes and Taussky (7) for the colorimetric determination of creatinine was used. Since twenty-four hour urine samples were not available, Shaffer's creatinine coefficient (8) was modified and defined as the concentration (milligrams/milliliter) of creatinine per kilogram of body weight. Pigment was measured as the per cent of absorption of 420 mu light by the clear urine sample as determined by a Coleman spectrophotometer. Phosphate was determined colorimetrically by the method of Fiske and Subbarow (9). Chloride was determined by the Mohr method of titration (10). Total carbohydrates were determined using Dreywood's reagent (11) and reported in terms of glucose. Ketonic substances were determined colorimetrically by a method originally described by Behre and Benedict (12) and modified for this study. Choline was determined by microbiological assay through use of the method described by Horowitz and Beadle (13).

Results and Discussion

Those items which exhibited substantial group differences were selected from the larger mass of data (14), and are presented in Table II. Data on pH, specific gravities, and volumes are also included in Table II, although these items showed no marked group differences. Standard statistical methods (number of standard errors as calculated using the standard deviation from the arithmetical mean) were employed (15) to determine the relative significance of each item examined. A difference between the overweight and the underweight groups which gave two or more standard errors was considered statistically significant, since at two standard errors the odds against the difference of the two means being due merely to chance is slightly greater than 20 to 1. In biological work in which small numbers of samples are involved it is customary to accept the 5% level (20 to 1) as being strongly indicative of statistical significance. The items studied are listed in descending order of significance in Table III. It is interesting to note that three of the items (pH, volume, specific gravity) did not show any marked group differences, thus lending greater credence to those differences observed.

When significant results are obtained in a study of the present kind, the problem remains to determine whether the observed differences are causes or results. However, regardless of which they are, some idea of the metabolic processes involved can be obtained. Hence it is desirable to examine the experimental data for any metabolic aberrations which may be involved in obesity.

TABLE II

Summary of Analytical Data Including the Items Showing Substantial Differences Between Overweight and Underweight Groups

Sample Number	Volume ml.	(Specific gravity − 1) x 10^3	pH	Modified Creatinine Coefficient	Pigment: Creatinine	Phosphate mg./ml.	Calcium mg./ml.	Phosphorus mg./ml.	Creatinine mg./ml.	Chloride mg./ml.	Boron μg./ml.	Choline μg./ml.	Copper μg/ml.
Underweight:													
20a	220	31	5.2		18.1	1.50	0.153	1.20	2.60	9.00	2.35	14.5	0.13
b	300	23	5.2	3.48	19.7	1.65	0.203	1.73	1.88	5.30	2.52	12.5	0.20
c	305	32	5.6		16.9	1.40	0.074	1.15	1.72	9.85	2.45	8.0	0.07
21a	170	31	5.4		14.8	2.45	0.245	2.15	3.12	7.15	1.81	12.0	0.13
b	235	28	5.6	4.46	15.6	1.65	0.422	1.42	2.56	7.25	1.92	5.2	0.15
22a	175	24	5.6		14.7	2.35	0.210	2.55	3.00	3.75	7.00	2.0	0.21
b	145	26	5.6	4.48	14.8	2.35	0.050	1.32	2.84	5.10	4.00	0.4	0.12
c	155	27	5.6		14.4	2.50	0.143	2.38	2.84	4.05	8.00	3.8	0.18
23a	215	25	5.3		12.1	1.90	0.170	1.87	2.40	5.95	2.60	16.8	0.14
b	190	30	5.4	4.84	12.0	1.65	0.164	1.68	3.00	6.30	2.72	14.5	0.14
c	190	28	5.4		10.1	2.30	0.275	2.08	2.68	5.45	2.40	0.9	0.20
24a	305	26	5.4		14.1	2.70	0.194	2.77	2.56	4.25	2.60	6.9	0.17
b	440	24	5.4	2.57	19.1	1.60	0.139	1.59	1.88	5.80	2.30	4.7	0.16
c	430	16	5.2		22.5	0.85	0.046	1.65	1.20	1.55	1.50	3.5	0.21
25a	205	24	5.7		17.6	1.50	0.104	1.50	1.88	5.15	8.40	7.5	0.14
b	160	32	5.5	4.67	13.4	1.65	0.245	1.32	2.24	5.75	4.50	7.1	0.11
26a	275	20	5.8		23.7	1.65	0.155	1.58	1.60	3.60	3.75	4.9	0.10
b	405	18	6.0	2.63	19.2	0.90	0.155	0.96	1.20	4.05	3.30	0.2	0.10
c	210	23	5.3		18.0	1.60	0.129	1.68	2.00	4.40	3.00	3.6	0.18
27a	185	21	5.3		16.2	1.65	0.063	1.62	2.04	5.30	3.90	10.2	0.12
b	255	20	5.4	2.80	14.1	1.25	0.042	1.62	1.84	3.10	4.10	8.5	0.30
c	265	15	5.5		17.5	1.05	0.030	1.56	1.48	2.40	2.10	9.6	0.20
28a	480	17	6.6		20.3	0.90	0.097	0.89	1.28	4.35	1.20	2.3	0.10
b	120	21	5.6	2.70	20.6	0.85	-- -----	-- ---	1.60	8.05	-- ---	4.3	-- ---
c	190	18	6.3		19.9	0.65	-- -----	-- ---	1.36	6.60	-- ---	2.5	-- ---
29a	230	30	5.7		22.3	1.75	0.174	1.50	2.24	7.55	2.75	4.0	0.12
b	235	30	5.6	3.49	20.5	1.40	0.210	1.19	2.00	8.50	3.30	2.0	0.12
c	215	27	6.0		18.5	1.20	0.275	1.03	1.84	10.25	2.00	2.0	0.12

TABLE II—(Continued)

Summary of Analytical Data Including the Items Showing Substantial Differences Between Overweight and Underweight Groups

Sample Number	Volume ml.	(Specific gravity − 1) x 10^3	pH	Modified Creatinine Coefficient	Pigment: Creatinine	Phosphate mg./ml.	Calcium mg./ml.	Phosphorus mg./ml.	Creatinine mg./ml.	Chloride mg./ml.	Boron μg./ml.	Choline μg./ml.	Copper μg/ml.
Overweight:													
10a	240	33	5.6		13.0	3.55	0.166	3.18	3.48	5.20	3.50	18.3	0.22
b	205	31	5.5	3.24	10.9	3.30	0.111	2.79	3.04	4.75	2.52	10.5	0.21
c	270	34	5.6		12.0	3.65	0.180	2.40	3.00	5.85	3.00	14.5	0.21
11a	105	32	5.8		12.5	5.90	0.093	3.80	3.84	2.90	3.00	22.5	0.24
b	115	25	5.7	4.12	8.7	4.25	0.147	3.62	5.32	3.00	3.55	19.5	0.20
c	97	28	5.5		9.4	3.85	0.217	3.36	5.52	3.40	5.20	1.3	0.31
12a	325	27	5.4		15.0	2.45	0.418	2.38	2.40	5.40	1.81	20.0	0.17
b	310	23	5.4	2.14	12.9	1.70	0.323	1.74	2.48	4.45	1.88	1.0	0.15
c	213	30	5.3		11.3	2.00	----	2.22	2.56	6.40	1.85	16.5	0.15
13a	475	16	5.4		22.8	0.55	0.069	1.00	1.14	2.85	2.90	3.3	0.15
b	430	31	5.3	1.56	16.5	2.05	0.222	1.49	2.40	6.55	3.90	6.3	0.11
c	490	28	5.2		18.4	1.05	0.267	1.17	1.36	6.60	2.00	4.9	0.11
14a	195	33	5.6		21.5	1.80	0.142	1.77	2.60	6.50	2.48	8.3	0.17
b	375	25	5.6	2.52	22.2	1.05	0.080	1.17	1.48	4.85	2.20	9.3	0.14
c	160	35	5.3		13.0	2.20	0.215	1.85	3.84	8.75	2.40	13.0	0.11
15a	245	25	4.9		12.1	1.80	0.205	2.00	2.24	4.30	3.70	5.0	0.19
b	170	26	5.0	2.03	13.2	3.65	0.348	1.95	2.12	5.00	1.80	3.0	0.15
c	220	34	4.9		11.5	2.25	0.320	2.25	3.12	3.40	2.50	5.1	0.18
16a	350	18	5.5		17.0	0.70	0.094	0.97	1.24	4.55	2.90	1.9	0.12
b	475	10	5.9	1.26	12.5	0.80	0.039	0.80	0.64	2.65	0.72	0.7	0.13
c	365	24	5.8		14.6	1.15	----	----	1.64	6.10	----	0.8	----
Overweight Mean	277	27.0	5.45	2.41	14.3	2.37	0.230	2.10	2.64	4.93	2.69	8.9	0.17
Underweight Mean	248	24.5	5.60	3.65	17.2	1.60	0.160	1.62	2.10	5.71	3.32	6.2	0.15

There are some nineteen items for consideration in this regard, (excluding duplication due to the use of two methods for phosphate determination), four of which are statistically significant.

TABLE 3

Summary of Results of Urinary Studies of Overweight and Underweight Groups in Order of Decreasing Significance

Factor	Factor was higher in Over-weight Group	Factor was higher in Under-weight Group	No. of Standard Errors	Odds*
Modified Creatinine Coefficient	—	×	2.76	173:1
Pigment/Creatinine	—	×	2.71	148:1
Phosphate	×	—	2.49	78:1
Calcium	×	—	2.34	52:1
Phosphorus	×	—	2.23	38:1
Creatinine	×	—	1.86	15:1
Boron	—	×	1.51	7:1
Choline	×	—	1.50	6.5:1
Chloride	—	×	1.50	6.5:1
Copper	×	—	1.38	5:1
Ketonic Substances	×	—	0.95	2:1
Volume	×	—	0.84	1.5:1
Iron	×	—	0.73	1:1
Specific Gravity	×	—	0.71	1:1
Potassium	×	—	0.67	1:1
Magnesium	×	—	0.60	< 1:1
pH	—	×	0.55	< 1:1
Sodium	×	—	0.43	< 1:1
Total Carbohydrates	×	—	0.12	< 1:1
Pigment	—	—	0	-----

*Odds against the difference between the two means being due merely to chance.

Since the excretion of such substances as sodium, potassium, and magnesium ions were approximately the same in the two groups, it would appear that the members of the two groups had a similar mineral intake. It is very unlikely that anyone in this age group would store large amounts of calcium and phosphorus. Inasmuch as the underweight persons studied here excreted less of these two elements in the urine, it follows that more must have been excreted through the feces. Other work which is reported in this bulletin (16) indicates that different strains of rats show marked differences in urinary phosphate excretion. Genetic factors may similarly be operative in human phosphate metabolism.

Speculation with respect to this situation leads to the idea that obese people, who have a large amount of fat which is in constant dynamic equilibrium, might require larger amounts of phospholipides for fat transport. The presence of greater amounts of phospholipides would mean that normal catabolic processes would lead to greater phospholipide breakdown, with correspondingly higher amounts of breakdown products in the blood and finally in

the urine. Higher phosphate values in the urine of overweight persons are in line with this idea as are also the higher choline values.

The elevated urinary excretion of calcium in the overweight group may be the result of the fact that calcium and phosphorus are intimately related in metabolic processes. Another possible explanation for the increased calcium may be in the fact that calcium is involved in some obscure manner in fat metabolism, since in arteriosclerosis the deposition of calcium is preceded by a laying down of a layer of lipide material.

The relative values for the modified creatinine coefficient merely support well-established findings and probably indicate that the selection of the groups was performed in a valid manner. Drabkin (17,18) observed that the output of urinary pigment is independent of the diet, but bears a relation to the level of basal oxygen consumption and is, therefore, a product of endogenous metabolism. The ratio of pigment to creatinine as an index of basal metabolic rate was suggested by Ostow and Philo (19) and studied further by Vorzimer, *et al.* (20). The ratio of pigment to creatinine obtained in the present study indicates that the overweight group tends to have a lower basal metabolic rate than the underweight group.

The fact that the ketonic substances in urine were approximately the same in the two groups indicates strongly that the obese persons do not have a specific impairment in their ability to oxidize completely the fat acids.

Slightly elevated excretion of copper in the overweight group is of some interest in that copper deficiencies result in a deterioration of the lipoidal membranes of nerve cells (21), and it is possible that the catalytic effect of some material such as copper may eventually be shown to be intimately associated with conditions of obesity. Further work is necessary in order to elucidate and establish such relationships as actually exist.

Aside from their bearing upon the problem of obesity, various of the data clearly demonstrate how many commonplace urinary constituents are highly individualistic. While it is not practical to consider this aspect of the experimental work at great length in this paper, it is desirable to call attention to the magnitude of the many consistent individual differences that manifest themselves.

The data obtained are not sufficient to permit definite conclusions as to the etiology of obesity. However, further investigation of certain aspects, particularly of phospholipide metabolism, should bear fruitful results.

Summary

1. The following fourteen metabolic factors were measured and studied in the urine of a group of seven overweight and ten underweight individuals: potassium, sodium, boron, magnesium, iron, calcium, phosphorus, copper, chloride, creatinine, pigment, total carbohydrates, ketonic substances, and choline.

2. The following items showed sufficient differences between the two groups to be suggestive of the involvement of metabolic factors in obesity: modified creatinine coefficient, pigment to creatinine ratio, urinary phosphate excretion, and urinary calcium excretion.

3. The data suggest that obese people have characteristic metabolic differences involving calcium and phosphorus but that their ability to catabolize fats may be normal.

4. Highly characteristic individual metabolic differences were observed in the subjects studied.

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XXI. Metabolic Patterns of Schizophrenic and Control Groups

by

M. KENDALL YOUNG, JR., HELEN KIRBY BERRY, ERNEST BEERSTECHER, JR., AND JIM S. BERRY

Test Group and Controls: The eighteen male mental patients used in this study were free from any known "organic" disease, and all but one were diagnosed as suffering from dementia praecox. They ranged in age from 20 to 45 years, with the mean age being 31 years. Patients numbers 1 through 10 were described as hebephrenic; 11 through 14 were classified as simple type; 15 and 16 were paranoid; 17, in addition to being schizophrenic, suffered from epilepsy. Patient No. 18 was classified as manic depressive, depressed, and was included in this study because a number of workers consider this type of mental disorder biologically the same as dementia praecox.

The control groups were also free from any known organic disease and were from an age group similar to that of the patients.

Urine and saliva samples were collected on three successive days from both groups and refrigerated until the analyses could be made. Samples obtained immediately after the subjects arose in the morning were collected in order to have fairly representative samples.

Analyses: Creatinine was determined colorimetrically by the standard method of using alkaline picric acid (1).

The *carbohydrates* in both urine and saliva were determined spectrophotometrically using Dreywood's Reagent. The procedure described by Morris (2) was employed.

Magnesium in urine was determined colorimetrically by the formation of the titan yellow-magnesium hydroxide lake (3). The method reported by Gillam (4) was employed, using 0.2 ml. of the titan yellow solution rather than the ten drops which Gillam used.

The amino acids with the exception of histidine were determined on two-dimensional paper chromatograms by the method described by Polson (5). Several of these values were checked using the one-dimensional chromatographic method reported by Berry and Cain (6).

Urea and uric acid were determined on one-dimensional paper chromatographs as described in other portions of this Bulletin (7).

Histidine and diazonium-coupling compounds: Histidine and a number of unidentified substances present in urine were determined using one-dimensional paper chromatograms. Volumes of urine containing approximately 100 micrograms of creatinine were placed 1¼ inches apart, 1 inch from the bottom of an 11 x 18 inch sheet of Whatman No. 1 filter paper. The sheets were then developed in a solvent mixture consisting of 80 ml. of n-butanol, 20 ml. of glacial acetic acid, and 20 ml. of water using the ascending method described by Williams and Kirby (8). The sheets were removed from the solvent when it reached the top edge and were allowed to dry thoroughly at room temperature. Color development was achieved by the following treatment: The dried sheets were sprayed with 0.4% diazotized sulfanilic acid solution, allowed to dry, and then sprayed with a 10% solution of sodium carbonate to develop color. Figure 1 represents a composite chromatogram of the substances revealed by the diazo reagent, their relative size, and their positions. The concentrations, reported as spot area in square inches, may be found for histidine in Table I and for the unknown diazonium-coupling compounds in Table II.

Results and calculations: The data for the patients and the control groups are reported as the average of three or more samples per individual in Tables I and II. All the data, with the exception of salivary glucose and creatinine which are reported on a milligram per milliliter basis, are based on creatinine excretion. Creatinine was chosen as a basis because for healthy subjects it is the most constant excretory product for a given individual from day to day (9) and its use as a basis compensates for concentration differences in the urine samples. The validity of using this substance as a basis for calculation in the schizophrenic group is discussed below.

The standard deviation, σ, for control and schizophrenic groups, the ratios of the standard deviations, and the P values are reported wherever the calculations are practicable. The statistical data are calculated according to the methods outlined by Snedecor (10). The P values, however, are reported as $1 - n$, where n is the P value taken from Snedecor's Table of Values of t. Thus a P value of 0.99 indicates that the probability that the differences between control and schizophrenic groups is not due to chance occurrence, is 99 per 100.

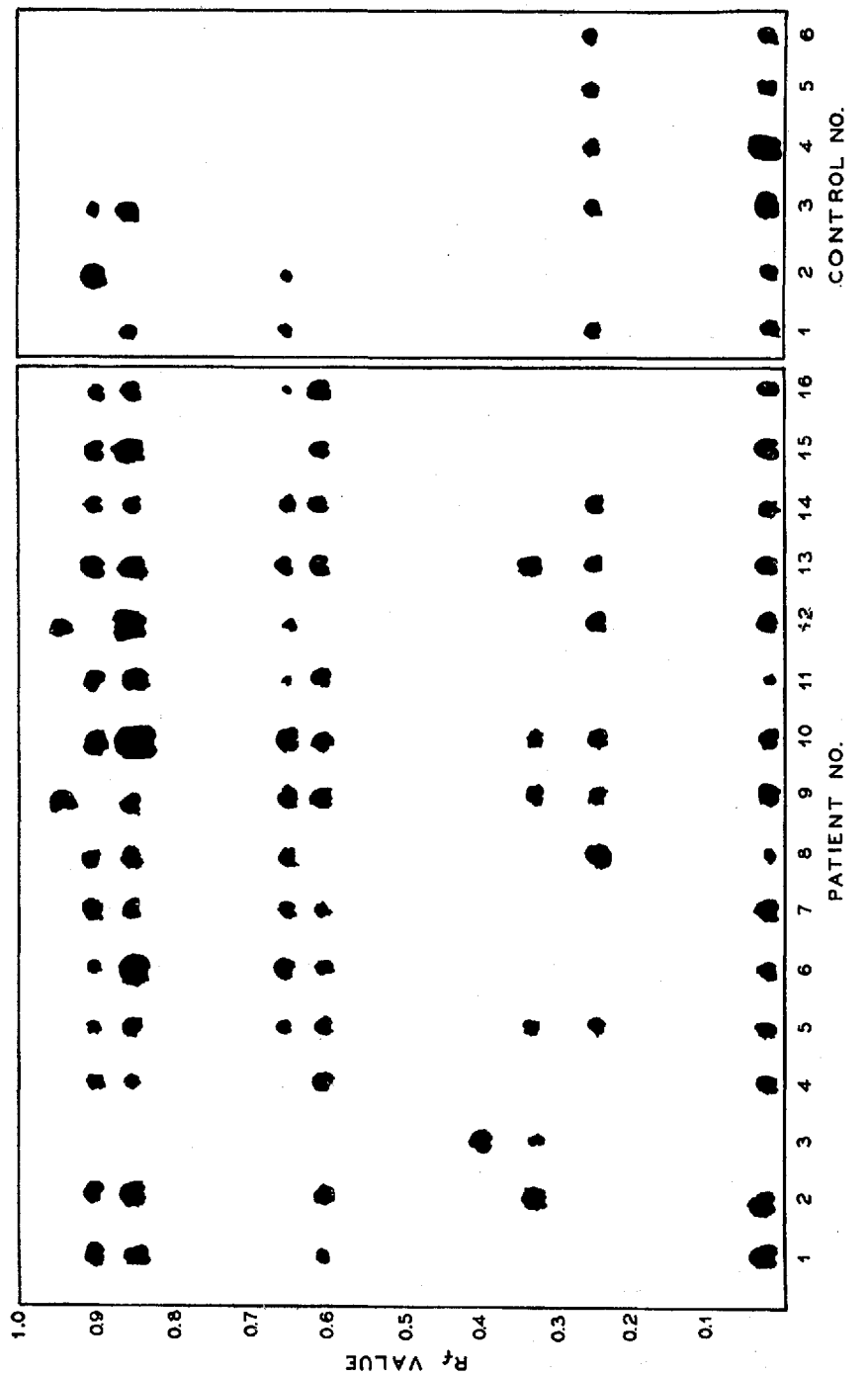

FIGURE I.

Diagram of Chromatogram of Urine of Mental Patients and Control Group.
(Solvent: Butanol-acetic acid-water. Developing reagent: Diazotized sulfanilic acid).

TABLE 1

Patient No.	Creatinine mg./ml.	Specific Gravity	Urea mg./mg.Cr.	Uric Acid mg./mg.Cr.	Glucose mg./mg.Cr.	Glutamic Acid mg./mg.Cr.	Serine mg./mg.Cr.	Glycine mg./mg.Cr.	Taurine mg./mg.Cr.	Alanine mg./mg.Cr.	Citrulline mg./mg.Cr.	Valine mg./mg.Cr.	Methionine sulfoxide mg./mg.Cr.	Histidine in ^{2}/100 μg.Cr.	Magnesium mg./mg.Cr.	Salivary Glucose mg./ml.
1	2.52	1.026	15	0.39	0.28	0.002	0.030	0.040	0.016	0.016	0.050	0.010	vis	0.43	0.065	2.70
2	2.66	1.027	14	0.37	0.40	0.000	0.034	0.034	0.015	0.019	0.049	0.004	0.000	0.40	0.072	0.34
3	0.59	1.010	12	—	0.37	0.000	0.050	0.050	0.000	0.020	0.100	0.000	0.000	0.00	0.140	0.27
4	1.62	1.018	15	0.28	0.28	0.000	0.050	0.060	0.020	0.030	0.060	0.000	0.000	0.34	0.084	0.00
5	3.10	1.037	16	0.52	0.42	0.005	0.026	0.023	0.006	0.012	0.020	0.013	vis	0.19	0.077	1.65
6	3.96	1.035	13	0.35	0.44	0.003	0.020	0.030	0.008	0.070	0.023	0.018	vis	0.28	0.053	0.76
7	1.89	1.026	14	0.16	0.33	0.002	0.030	0.040	0.013	0.020	0.025	0.004	0.002	0.38	0.066	1.62
8	1.93	1.021	19	0.36	0.39	0.005	0.026	0.036	0.000	0.030	0.040	0.000	vis	0.15	0.075	0.54
9	0.72	1.007	10	0.28	0.50	0.000	0.006	0.018	0.000	0.000	0.000	0.000	0.000	0.21	0.120	0.93
10	1.16	1.012	20	0.23	0.33	0.006	0.018	0.051	0.045	0.010	0.018	0.026	0.030	0.30	0.107	0.25
11	0.62	1.009	16	0.19	0.84	0.012	0.036	0.048	0.000	0.012	0.000	0.000	0.000	0.08	0.166	0.52
12	1.10	1.015	13	0.18	0.29	0.011	0.018	0.075	0.036	0.008	0.019	0.012	0.011	0.38	0.118	0.25
13	3.29	1.028	12	0.15	0.22	0.005	0.020	0.028	0.012	0.017	0.049	0.010	vis	0.44	0.070	0.32
14	3.84	1.031	13	0.26	0.32	0.021	0.019	0.045	0.022	0.019	0.033	0.008	0.012	0.28	0.041	—
15	2.56	1.026	11	0.39	0.52	0.021	0.050	0.050	0.012	0.006	0.000	0.008	0.012	0.23	0.094	0.36
16	1.64	1.026	22	0.50	0.55	0.008	0.060	0.060	0.012	0.000	0.000	0.000	0.015	0.22	0.092	0.00
17	1.35	1.009	13	0.15	0.59	0.015	0.014	0.032	0.035	0.009	0.022	0.000	0.000	—	—	—
18	1.31	1.017	23	0.21	0.56	0.013	0.024	0.054	0.037	0.021	0.054	0.020	0.000	—	—	—
Control No.																
1	1.72	1.011	—	—	0.34	0.003	0.008	0.018	0.011	0.006	0.015	0.002	0.001	0.36	0.037	0.14
2	2.98	1.026	—	—	0.23	0.000	0.018	0.027	0.011	0.006	0.015	0.000	0.005	0.35	0.039	0.21
3	1.94	1.025	—	—	0.40	0.000	0.014	0.014	0.000	0.009	0.029	0.000	0.008	0.49	0.083	0.18
4	1.66	1.008	—	—	0.12	0.000	0.000	0.008	0.000	0.007	0.010	0.000	0.000	0.32	0.025	0.22
5	3.78	—	—	—	0.20	—	—	—	0.000	—	—	—	—	—	0.034	0.49
6	1.47	1.015	—	—	0.33	0.003	0.000	0.005	0.000	0.013	0.034	0.000	0.000	0.53	0.066	0.33
7	3.57	—	—	—	0.20	0.000	0.012	0.022	0.032	0.014	0.035	0.000	0.000	—	0.031	0.42
8	1.79	—	—	—	0.43	0.000	0.005	0.015	0.006	0.006	0.019	0.000	0.008	—	0.095	0.33
9	2.26	—	—	—	0.35	0.000	0.000	0.006	0.000	0.005	0.013	0.000	0.000	—	0.050	—
10	2.12	—	—	—	0.32	0.004	0.018	0.018	0.000	0.014	0.040	0.000	0.000	—	0.078	—
11	2.56	—	—	—	0.44	—	—	—	—	—	—	—	—	—	0.044	—

Aver. Patients	1.99	1.016	15	0.29	0.42	0.007	0.030	0.043	0.016	0.018	0.031	0.007	0.004	0.27	0.090	0.70
Controls	2.35	-- -----	---	-- ---	0.30	0.001	0.008	0.015	0.007	0.009	0.023	0.000	0.002	0.41	0.053	0.29
Stand. Dev. Patients	1.07	-- -----	---	-- ---	0.15	0.007	0.016	0.015	0.014	0.016	0.026	-- -----	0.008	0.13	0.033	0.75
Controls	0.78	-- -----	---	-- ---	0.10	0.002	0.008	0.007	0.011	0.004	0.011	-- -----	0.005	0.09	0.024	0.12
σPatients/σControls	2.2	-- -----	---	-- ---	1.5	3.5	2.0	2.1	1.3	4.0	2.4	-- -----	1.6	1.4	1.4	6.3
P=	0.66	-- -----	---	-- ---	0.34	0.99	0.99	0.99	0.91	0.90	0.70	-- -----	0.96	0.96	0.99	0.85

Discussion

The data which appear in the tables, when analyzed statistically, show some interesting and significant differences between the two groups studied. If the arbitrary assumption is made that a *P* value equal to or greater than 0.90 is statistically significant, it is notable that the excretion values for magnesium, a number of the diazonium-coupling compounds and all the amino acids except citrulline, possess significance. Several other constituents approach this value of 0.90 and probably are also significant.

TABLE II

Imidazole Derivatives and Related Compounds
Area (in^2)/100 g. Creatinine

Patient No.	Rf = .25	.32	.60	.65	.85	.90	.92
1	0.00	0.00	0.09	0.00	0.38	0.16	0.00
2	0.00	0.20	0.12	0.00	0.35	0.15	0.00
3	0.00	0.04	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.19	0.00	0.11	0.10	0.00
5	0.12	0.15	0.08	0.12	0.32	0.14	0.00
6	0.00	0.00	0.12	0.13	0.51	0.14	0.00
7	0.00	0.04	0.07	0.13	0.18	0.16	0.00
8	0.34	0.00	0.00	0.12	0.25	0.11	0.00
9	0.00	0.00	0.26	0.11	0.21	0.00	0.26
10	0.16	0.12	0.26	0.23	0.83	0.32	0.00
11	0.00	0.00	0.25	0.03	0.44	0.32	0.00
12	0.15	0.00	0.00	0.07	0.59	0.00	0.25
13	0.15	0.22	0.16	0.16	0.41	0.30	0.00
14	0.17	0.00	0.15	0.13	0.11	0.10	0.00
15	0.00	0.00	0.18	0.00	0.44	0.21	0.00
16	0.00	0.00	0.28	0.05	0.20	0.17	0.00
17	----	----	----	----	----	----	----
18	----	----	----	----	----	----	----
Control No.							
1	0.15	0.00	0.00	0.13	0.18	0.00	0.00
2	0.00	0.00	0.00	0.08	0.00	0.34	0.00
3	0.14	0.00	0.00	0.00	0.37	0.10	0.00
4	0.23	0.00	0.00	0.00	0.00	0.00	0.00
5	0.20	0.00	0.00	0.00	0.00	0.00	0.00
6	0.24	0.00	0.00	0.00	0.00	0.00	0.00
Aver.							
Patients	0.07	0.05	0.14	0.08	0.33	0.15	0.00
Controls	0.16	0.00	0.00	0.03	0.09	0.07	0.00
P =	0.93	----	----	0.76	0.98	0.82	----

The lowered creatinine average for mental patients and the *P* value of 0.66 raise the question of the validity of using creatinine as a basis for correcting for urine concentration. An inspection of the standard deviation values demonstrates that for creatinine excretion, just as for the excretion of the other substances measured, there is a much greater day to day variation in the schizophrenic group than in the controls. However, if the difference in the two

groups is compensated by multiplying the individual control values by the ratio 2.35/1.99 and recalculating the *P* values, it is found that the new values are not markedly different from those given in the table; so the apparent invalidity of the creatinine basis can be discounted.

A survey of the literature pertaining to the biochemical aspects of the schizophrenic syndrome discloses a confusing and contradictory picture. Bellak (11), in his exhaustive survey of the subject, has even stated that "its only consistent difference from the normal lies in the greater variability of values for almost any factor investigated." The increased variability is amply confirmed in the data presented in Table I; the ratios of standard deviations of mental patients to controls range from 1.3 to 6.3 with not a single value less than unity.

The study of urinary magnesium excretion among mental patients has not been previously reported, but the *P* value and the comparative group averages show that excretion of this ion is almost certainly considerably higher among schizophrenics than in the normal population. This argues for altered magnesium utilization, and this theory offers several attractive speculative approaches to the problem of its relation to mental disease. The ratio of calcium to magnesium ions has long been known to have an intimate connection with tissue irritability; an increase in this ratio is associated with a rise in irritability. In addition, the deprivation of magnesium gives rise to vasodilation and hyperexcitability of the nervous system in rats, similar in symptomology to tetany in calcium deficient animals (12). Recent work has indicated that magnesium is required as an activator for enolase (13) and carboxylase (14) and is thus implicated in the metabolism of carbohydrates. Considerable attention has been devoted to anomalies in carbohydrate metabolism in dementia praecox (15), the principal inadequacy noted being delayed metabolism of blood sugar following intravenous glucose injection (16). The decreased action of the enzymes of the carbohydrate cycle due to magnesium depletion could well contribute to this effect. The increased excretion of this important constituent suggests further studies, especially with regard to the relation of blood levels to urinary levels, to determine its possible connection to the etiology of dementia praecox.

With regard to the possible relation of the urinary amino acids to schizophrenia, the foundation is more tenuous. With the exception of histidine, all are excreted in markedly higher amounts. Unpublished studies made at this laboratory have indicated that high

levels of urinary excretion of these amino acids are found in normal women. This finding, coupled with the report by Hoskins and Pincus (17) that the androgen to estrogen ratios in male schizophrenics are very markedly depressed from normal values, argues that lowered androgen production associated with testicular dysfunction (18) in dementia praecox might be correlated with the observed increased amino acid excretion. Histidine excretion, on the other hand, is somewhat lower for the group of mental patients. This could be associated with the increased excretion of unusual substances such as some of the diazonium-coupling compounds or others not detected. Alternatively, it could be related to the existence of the higher histamine blood levels reported by Strengers and Gooszen (19).

The nature and significance of the diazonium-coupling compounds are totally unknown. However, the very fact that some of these substances were found solely in the samples from the mental patients indicates that they are potentially important and certainly interesting in the study of dementia praecox. Koessler and Hanke (20, 21) indicate that among the urinary constituents which react with diazotized sulfanilic acid are histidine, imidazole propionic acid, imidazole lactic acid, and imidazole acetic acid. These authors mention a study of the diazo reaction in the urine of normal and pathological cases in which they found an increased excretion of imidazole derivatives in pathological states. The need for further study is most apparent here; until the identity of these compounds can be determined, no speculation is feasible concerning their relation to the schizophrenic state, despite the striking contrast exhibited by the two groups.

The remaining substances reported in the table show only slight differences between the two groups, and the *P* values are low. Salivary gucose alone seems to have some significance, the nature of which is not known. It might be expected, in view of the disturbances in carbohydrate metabolism which have been reported, that the urinary excretion might reflect these differences, but aside from a slightly higher excretion, which is not significant, and a greater variability among the patients, the difference is slight.

The data presented support the thesis that dementia praecox is essentially a disease of physiological deficiency and demonstrate the existence of a biochemical pattern strikingly different from that of normal controls. More than all, this work has shown the need for additional concentrated and systematized study, on a sound scientific basis, of the problem of mental disease. The aid of power-

ful, facile tools has made this study practicable; the magnitude of the problem, both from a scientific and from a sociological viewpoint, makes it necessary.

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XXII. Exploration of Metabolic Patterns in Mentally Deficient Children

by

LOUISE CAIN

Significant differences in metabolic patterns have been found in this laboratory to exist when alcoholics were compared with non-alcoholics (1), when obese individuals were compared with lean individuals (2) and when mental patients were compared with well individuals (3). The study here presented was made to explore the possible significant differences between the patterns of mentally deficient children and those with normal faculties. This particular investigation is limited, however, to a study of the urinary excretion patterns.

Thirteen mentally deficient children, 7 to 11 years of age, from the Austin State School were chosen as subjects. These were selected by the medical staff to be as free as possible from physical defects. For comparison, ten control children, ages 5 to 11, were studied in a similar manner. Three morning urine samples were obtained from each individual child. The mentally deficient children were placed in the hospital ward overnight to insure proper urine collection.

Amino acid determinations were made according to the methods of Berry and Cain (4). Three two-dimensional sheets were run on each individual urine sample, using aliquots of urine of 20 μl., 40 μl., and the amount containing 50 μg. of creatinine. When the amino acid concentrations were low enough so that they were not detected on the sheets involving the smaller aliquots, the values obtained on the sheets involving larger aliquots were used for computation. Otherwise the values obtained from the three sheets were averaged.

Most of the amino acids were determined by direct comparison with standards employing known amounts of the amino acids in question. Phenylalanine, however, was determined on the basis of its detectability at 5 μg. This amount is necessary to make a clearly defined spot when, as under ordinary conditions, leucine is also present and migrates to the same position. Proline and methionine also were not included in the standards. Proline was evaluated on the basis of its detectability at 7 μg. and methionine by comparison with alanine standards. An unidentified ninhydrin-positive blue spot (Rf value in phenol .21, in lutidine .78) was

determined only on a relative basis, but values were assigned to it on the arbitrary assumption that it is detectable at 1 μg. Histidine was determined by the method previously described in this bulletin (p. 77).

Creatinine was determined colorimetrically in all samples by the standard picrate method (5), phosphorus by the colorimetric method of Fiske and Subbarow (6) and carbohydrates by the colorimetric method employing Dreywood's anthrone reagent (7).

Creatine, uric acid and urea were determined by paper chromatography in accordance with methods described in this bulletin (8,9,10) except that in the case of urea a modification was made. Instead of using sodium hypochlorite as the color developing agent, the sheet after resolution with phenol solvent was thoroughly dried and then developed with 1% *p*-dimethylaminobenzaldehyde in 10% HCl-alcohol (p. 30).

TABLE I

Additional Urine Chromatograms

Aliquot of urine	Color developing agent	Substances detected
1. ≡ 100 μg. creatinine	diazotized sulfanilic acid	Unidentified spots: Rf .63 — yellow Rf .65 — pink Rf .80 — orange-yellow Rf .89 — purple
2. ≡ 60 μg. creatinine	ferric chloride	Phosphate Unidentified spots: Rf .30 — blue-gray Rf .62 — blue-gray
3. ≡ 40 μg. creatinine	*p*-dimethylaminobenzaldehyde	Unidentified spots: Rf .36 — purple Rf .51 — pink Rf .75 — purple
4. ≡ 40 μg. creatinine	ferricyanide-nitroprusside reagent	No arginine detectable No spots other than those already detected
5. ≡ 40 μg. creatinine	aniline acid phthalate	No spots corresponding to sugars found
6. ≡ 40 μg. creatinine	2,6-dichloroquinone chloroimide	urea uric acid creatinine
7. ≡ 40 μg. creatinine	bromcresol green	lactic acid* hippuric acid* tartaric acid*
8. ≡ 40 μg. creatinine	bromine	Unidentified spots: Rf .52 — fluorescent
9. ≡ 50 μg. creatinine	picric acid	Rf .10 — creatine Rf .30 — creatinine Unidentified spots: Rf .48 — gray-green† Rf .35 — yellow

*These were determined by measuring the area of the spots using a polar planimeter.
†This unidentified spot was evaluated on the basis of color intensity and was rated from 0 to 4+.

TABLE II

Data with Respect to Items which Appeared to Show Differences

Mentally Deficient Children	Age	Sex	"I.Q."	Methionine sulfoxide† mg./mg. Cr.	Tyrosine mg./mg. Cr.	Proline mg./mg. Cr.	Green-Gray spot (picric acid) mg./mg. Cr.	Blue spot (ninhydrin) mg./mg. Cr.	Histidine mg./mg. Cr.	Taurine mg./mg. Cr.	Lysine mg./mg. Cr.	Phenyl alanine mg./mg. Cr.
1	9	F	23	0.067	0.066	0.239	0.33	0.009	0.355	0.059	0.055	0.123
2	9	M	37	0.126	0.012	0.117	0.66	0.000	0.084	0.000	0.040	0.065
3	7	F	36	0.226	0.083	0.069	1.66	0.019	0.260	0.049	0.140	0.119
4	8	F	31	0.181	0.036	0.253	0.66	0.025	0.110	0.077	0.022	0.123
5	8	F	30	0.031	0.000	0.131	0.66	0.029	0.096	0.000	0.037	0.038
6	11	F	61	0.028	0.026	0.090	2.33	0.013	0.211	0.037	0.031	0.047
7	7	F	70	0.043	0.021	0.061	0.33	0.007	0.121	0.036	0.007	0.031
8	10	F	62	0.028	0.007	0.149	0.33	0.000	0.155	0.056	0.010	0.060
9	11	F	42	0.136	0.014	0.126	1.00	0.012	0.048	0.022	0.019	0.086
10	11	M	22	0.070	0.047	0.058	1.33	0.008	0.126	0.070	0.033	0.040
11	9	M	52	0.023	0.000	0.058	2.00	0.007	0.060	0.000	0.020	0.102
12	10	M	68	0.058	0.047	0.000	1.33	0.016	0.184	0.023	0.029	0.072
13	8	M	23	0.101	0.064	0.120	3.33	0.035	0.167	0.000	0.068	0.173
Ranges				0.023–0.226	0.000–0.083	0.000–0.253	0.33–3.33	0.000–0.035	0.06–0.355	0.000–0.077	0.007–0.14	0.031–0.173
Controls												
14	9	F	125	0.055	0.000	0.000	0.00	0.012	0.023	0.037	0.033	0.000
15	9	F	119	0.024	0.025	0.052	0.33	0.007	0.153	0.060	0.014	0.099
16	9	F	84	0.053	0.019	0.187	0.00	0.007	0.181	0.060	0.056	0.086
17	8	F	108	0.053	0.000	0.000	1.00	0.000	0.152	0.038	0.047	0.142
18	5	F	*	0.040	0.000	0.073	0.66	0.008	0.057	0.006	0.000	0.000
19	7	M	93	0.046	0.036	0.147	0.00	0.023	0.155	0.040	0.000	0.114
20	10	M	99	0.030	0.039	0.000	1.33	0.000	0.050	0.030	0.030	0.035
21	7	M	107	0.032	0.022	0.000	0.66	0.000	0.115	0.052	0.030	0.000
22	9	M	93	0.052	0.014	0.000	0.66	0.010	0.161	0.038	0.010	0.079
23	11	M	103	0.042	0.016	0.000	0.00	0.007	0.087	0.065	0.041	0.036
Ranges				0.024–0.055	0.000–0.039	0.000–0.187	0.00–1.33	0.000–0.023	0.023–0.181	0.006–0.065	0.000–0.056	0.000–0.142

*Preschool age—not determined.
†See footnote p. 75.

Seven additional one dimensional chromatograms (nos. 1–7, Table I) were run on each sample using butanol-acetic acid solvent; one additional chromatogram (No. 8, Table I) was made on each sample using isobutyric acid and one (No. 9, Table I) using butanol-ethanol.

The aliquots of urine used, the color developing agents employed and the substances determined in these nine sets of chromatograms are listed in Table I.

TABLE III

Statistical Analysis of Differences between Control and Mentally Deficient Groups

	Average Excretion mg./mg. Cr.	Standard deviation	Standard error	No. of Standard errors	Odds*
Methionine sulfoxide					
Controls	0.042	0.024			
Mentally deficient	0.087	0.068	0.011	3.9	10,390 to 1
Tyrosine					
Controls	0.017	0.018			
Mentally deficient	0.035	0.029	0.005	3.6	3,142 to 1
Proline					
Controls	0.040	0.077			
Mentally deficient	0.113	0.112	0.023	3.1	515.7 to 1
"Green-Gray Spot" Rf .48					
Controls	0.45	0.80			
Mentally deficient	1.15	1.10	0.23	2.6	106.3 to 1
"Blue Spot" (ninhydrin)					
Controls	0.008	0.011			
Mentally deficient	0.014	0.016	0.003	1.9	16.4 to 1
Histidine					
Controls	0.112	0.084			
Mentally deficient	0.152	0.092	0.021	1.9	16.4 to 1
Taurine					
Controls	0.043	0.030			
Mentally deficient	0.033	0.033	0.007	1.37	5.19 to 1
Lysine					
Controls	0.027	0.026			
Mentally deficient	0.039	0.033	0.008	1.4	5.19 to 1
Phenyl alanine					
Controls	0.059	0.096			
Mentally deficient	0.078	0.075	0.021	.89	1.12 to 1

*Odds against the differences between the two averages being due merely to chance.

In Table II are summarized the data with respect to all the items on all the chromatograms for which by inspection it appeared that there might be significant differences between the mentally deficient and the control groups. It may be noted that glycine, serine, alanine, citrulline, valine, leucine, aspartic acid, glutamic acid, threonine, methionine, cysteic acid, lactic acid, hippuric acid, tartaric acid, phosphate, carbohydates, uric acid, urea, creatinine and creatine, all of which were determined, are not listed in Table II, because in these cases the differences, if present, were small. The same statement holds for most of the unidentified spots.

In Table III is summarized the statistical analysis of the items included in Table II. It will be noted that for four items there appear to be statistically valid differences between the controls and the mentally deficient children according to conventional standards for biological work. For the two other items the differences are such as to indicate that they may be significant.

TABLE IV

Statistical Analysis of Differences Between Morons and Imbeciles

	Average Excretion mg./mg. Cr.	Standard deviation	Standard error	No. of Standard errors	Odds*
Methionine sulfoxide					
Imbeciles	0.115	0.068	0.016	5.2	1,744,000 to 1
Morons	0.033	0.028			
Carbohydrates					
Imbeciles	2.20	0.889	0.212	5.2	1,744,000 to 1
Morons	1.10	0.328			
Lysine					
Imbeciles	0.052	0.029	0.008	3.8	6,915 to 1
Morons	0.020	0.024			
Creatine					
Imbeciles	0.37	0.235	0.062	3.1	515 to 1
Morons	0.18	0.149			
Tyrosine					
Imbeciles	0.046	0.040	0.011	2.5	79.5 to 1
Morons	0.020	0.023			
Threonine					
Imbeciles	0.034	0.031	0.011	1.8	12.9 to 1
Morons	0.022	0.028			
Blue Spot					
Imbeciles	0.017	0.016	0.005	1.7	10.2 to 1
Morons	0.009	0.014			
Proline					
Imbeciles	0.131	0.115	0.033	1.38	5.2 to 1
Morons	0.085	0.093			
Phenyl alanine					
Imbeciles	0.087	0.076	0.024	1.1	2.7 to 1
Morons	0.062	0.070			
Glutamic acid					
Imbeciles	0.006	0.007	0.003	.80	1.4 to 1
Morons	0.004	0.008			

*Odds against the difference between the two averages being due merely to chance.

In the course of studying the data it appeared that the patterns of the morons and imbeciles differed significantly from each other. In Table IV is summarized the statistical analysis of the data with respect to ten items for which differences appeared probable. It will be noted that there are five significantly different items; in the majority of these the differences appear highly significant.

In the data assembled in both Tables III and IV the interesting fact should be noted that in general the greater excretion is associated with a higher degree of mental deficiency. In Table III eight of the nine items (taurine is the exception) are excreted in larger amounts by the mentally deficient than by the control children. For all of the significantly different items, the excretion by the mentally deficient children averages more than double that of the control children. In Table IV every one of the ten items is excreted in larger amount by the imbeciles than by the morons.

An interesting observation has to do with the carbohydrate excretion by controls, by morons and by imbeciles. Curiously carbohydrate excretion by the control group gives an average intermediate between that of the imbecile and moron groups. The probability of a significant difference between the imbeciles and morons is nearly 2 million to 1. Because the morons have lower excretion than the controls, however, the probability of a significant difference between the controls and the mentally deficient group as a whole is only 10 to 1. The findings with respect to carbohydrate excretion are summarized in Table V.

TABLE V

Statistical Analysis of Carbohydrate Excretion

	Average Excretion mg./mg. Cr.	Standard deviation	Standard error	No. of Standard errors	Odds*
Imbeciles	2.20	0.889	0.212	5.2	1,744,000 to 1
Morons	1.10	0.328			
Controls	1.46	0.664	0.218	3.4	1,483 to 1
Imbeciles	2.20	0.889			
Controls	1.46	0.664	0.164	2.2	35 to 1
Morons	1.10	0.328			
Controls	1.46	0.664			
Mentally deficient	1.77	0.775	0.173	1.7	10.2 to 1

*Odds against the difference between the two averages being due merely to chance.

A suggestive fact is that from inspecting the data with respect to the excretive patterns of the control individuals it was noted the pattern of one individual, No. 16, was different from the others in the control group and in some respects resembled the patterns of the

individuals in the mentally deficient group. This observation was made *before* the "I.Q." values for the control children had been collected; in fact this observation led us to collect these additional data which are included in Table II. It was found that this child not only had the lowest "I.Q." of any in the control group but was considered to be a special problem. In spite of relatively affluent surroundings, she had demonstrated antisocial behavior with respect to property rights.

In Table VI are presented, partly because of this special case and partly because of other data, a summary of the excretions for twenty-nine substances for which quantitative data are available. It may be noted that the imbecile group excreted *more* than the modified control group in 24 cases and *less* in 5. The moron group

TABLE VI

Summary of Excretion Averages

	Control Av. (Omitting 16) "I.Q." 95–125 mg./mg. Cr.	Imbecile Av. "I.Q." 22–42 mg./mg. Cr.	Moron Av. "I.Q." 51–70 mg./mg. Cr.	No. 16 Av. "I.Q." 84 mg./mg. Cr.
Alanine	0.040	0.048	0.056	0.048
Aspartic acid	0.005	0.006	0.006	0.015
Blue spot (Ninhydrin)	0.008	0.017	0.009	0.007
Citrulline	0.046	0.054	0.048	0.049
Cysteic acid	0.008	0.006	0.006	0.027
Glycine	0.074	0.082	0.080	0.076
Glutamic acid	0.003	0.006	0.004	0.000
Histidine	0.101	0.147	0.141	0.181
Leucine	0.017	0.024	0.023	0.040
Lysine	0.024	0.052	0.020	0.056
Methionine	0.013	0.006	0.007	0.008
Methionine sulfoxide	0.040	0.115	0.033	0.053
Phenyl alanine	0.056	0.087	0.062	0.086
Proline	0.021	0.131	0.085	0.187
Serine	0.067	0.070	0.057	0.076
Taurine	0.041	0.035	0.030	0.060
Threonine	0.031	0.034	0.022	0.060
Tyrosine	0.017	0.046	0.020	0.019
Valine	0.018	0.023	0.023	0.040
Carbohydrates	1.300	2.200	1.100	2.600
Creatine	0.31	0.37	0.18	0.26
Creatinine*	1.07	1.12	1.03	1.27
Green spot†	0.52	1.20	1.27	0.000
Hippuric acid††	0.15	0.28	0.19	0.31
Lactic acid††	0.17	0.24	0.18	0.27
Phosphorus	0.777	0.800	0.740	1.033
Tartaric	0.25	0.31	0.22	0.32
Urea	15.9	15.1	15.9	14.1
Uric acid	0.214	0.210	0.213	0.179

*Creatinine values represent milligrams per milliliter.
†Green spot values determined by color intensity.
††Hippuric acid and lactic acid values represent spot areas measured in square inches.

averaged *more* in 16 cases, *less* in 12 and the same in 1. Individual No. 16 averaged *more* in 23 cases and *less* in 6. These data are highly suggestive of the desirability of collecting this type of information with respect to human individuals, and studying its relationship to problems of human mentality and behavior. It should be obvious that such studies will probably not yield results that will be readily interpretable in a simple manner.

Summary

An exploratory study of the urinary excretion patterns of 13 mentally deficient and 10 control children have yielded results of a highly suggestive nature. It appears on the basis of the investigation up to this point that moron, imbecile, and control groups each exhibit excretion patterns which are distinctive in several respects. The findings also suggest the desirability of further investigation of these individual patterns in the case of so-called normal individuals, because of the probability that the findings may throw light on mental and behavior problems.

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