REFERENCES

Bailey, K. (1942). *Biochem. J.* **36**, 121.
Bailey, K. (1949). *Biochem. J.* **45**, 479.
Bailey, K. & Perry, S. V. (1947). *Biochim. biophys. Acta*, **1**, 506.
Banga, I. (1942). *Stud. Inst. med. Chem. Univ. Szeged*, **1**, 27.
Biro, N. A. & Szent-Györgyi, A. (1949). *Hung. acta physiol.* **2**, 120.
Engelhardt, V. A. & Lyubimova, M. N. (1939). *Nature, Lond.*, **144**, 668.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Herrmann, H. & Nicholas, J. S. (1948). *J. exp. Zool.* **107**, 177.
Herrmann, H., Nicholas, J. S. & Vosgian, M. E. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 454.
Kielley, W. W. & Meyerhof, O. (1948). *J. biol. Chem.* **176**, 591.
Kielley, W. W. & Meyerhof, O. (1950). *J. biol. Chem.* **183**, 391.
Laki, K. (1943). *Stud. Inst. med. Chem. Univ. Szeged*, **3**, 16.
Lyubimova, M. N. & Engelhardt, V. A. (1939). *Biochemistry, Leningr.*, **4**, 716.
Mommaerts, W. F. H. M. & Seraidarian, K. X. (1947). *J. gen. Physiol.* **30**, 401.
Needham, D. M. (1942). *Biochem. J.* **36**, 113.
Perry, S. V. (1952). *Biochim. biophys. Acta* (in the Press).
Robinson, D. S. (1952*a*). **52**, 621.
Robinson, D. S. (1952*b*). **52**, 628.
Szent-Györgyi, A. (1947). *Chemistry of Muscle Contraction.* New York: Academic Press.

The Amino-Acids and other Ampholytes of Urine

1. A GENERAL METHOD OF ISOLATION

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The nitrogenous constitutents of urine have been studied by many workers since the earliest days of biochemistry. Various methods have been devised for the estimation of the amino-acids and other nitrogenous components, but the actual isolation of the large variety of substances present in relatively small amounts has proved to be tedious and time-consuming. The invention of paper chromatography and the production of the newer synthetic ion-exchange resins has now provided a means whereby further studies of the nitrogenous substances which occur in urine have become more practicable. New interest in such methods has been stimulated by the discovery that in the routine examination of normal and pathological urines by paper chromatography carried out by Dent (1947, 1948) and his co-workers during the past 5 years, ninhydrin-reacting spots have been observed which could not be ascribed to any of the known naturally occurring amino-acids. Moreover, in almost every case, the positions of the unknown spots on the paper chromatograms were unchanged after hydrolysis by acid and this indicated that the unknown constituents were probably amino-acids and not peptides. Several of these substances have now been isolated from urine and preliminary communications have been published (Crumpler, Dent, Harris & Westall, 1951; Dent, Fowler & Walshe, 1951; Searle & Westall, 1951). The early stages of the method used for the isolation of these amino-acids were essentially the same in each case, and in order to avoid unnecessary repetition the general method of separation will be described in this paper, whilst the details of the isolation of the individual substances will be given in later papers of this series.

The procedure employed has evolved from studies on displacement chromatography on the synthetic ion-exchange resins Zeo-Karb 215 and De-Acidite E (Partridge & Westall, 1949; Partridge & Brimley, 1949). The fractionation of the amino-acids of acid-hydrolysed proteins of egg white has been described (Partridge, 1949) and further application has led to the isolation of γ-aminobutyric acid from beetroots (Westall, 1950*a*) and for the preparation in quantity of glutamine from the same source (Westall, 1950*b*). The application of the method, now modified by using the anion-exchange resin Dowex 2 instead of De-Acidite E, to the isolation of amino-acids from urine has not, so far, been described in detail.

General principles

The direct application of diluted urine to the Zeo-Karb columns resulted in the production of a gel within the column which disturbed the even flow of the liquid. This was due partly to traces of protein but also to some other ultrafilterable substance.

This disturbance is eliminated by evaporating the urine to small volume under reduced pressure and then adding 3 vol. of ethanol. The precipitate which forms is removed by filtration and washed with 80% (v/v) aqueous ethanol. No loss of amino-acids is incurred and a proportion of the inorganic salts is removed, together with the interfering substances. The ethanol in the filtrate is removed by evaporation under reduced pressure and the resultant syrup is diluted with water to twice the volume of the original urine. The passage of this solution through the system of Zeo-Karb columns enables the inorganic acids, organic acids, sugars and all substances including urea not functioning as cations, to be eliminated, since they are all carried away in the effluent. Certain organic ampholytes which carry a strongly acidic group, e.g. taurine, are not retained. Ca^{2+}, K^+ and Na^+ are retained at the top of the first column with the concomitant release of an equivalent number of H^+. This causes the aqueous phase within the columns to become strongly acidic and under these conditions the capacity of the resin for the retention of cations is low (Partridge & Westall, 1949). Obviously the initial precipitation with ethanol only partially removes the inorganic salts, and the size of the columns used in the experiment has to be unduly large for the collection of the relatively small amounts of amino-acids present. However, after the primary separation, subsequent fractionations can be carried out on much smaller columns since, in the absence of an excess of H^+, the organic bases and ampholytes are retained in much larger amounts—equivalent to 1·5–2·5 m-equiv./g. dry resin.

Subsequent treatment of the Zeo-Karb columns with 0·2N-ammonia displaces only those bases which are weaker than ammonia; the metal cations and some strong organic bases, as well as the basic amino-acids arginine and lysine, remain on the resin column. The effluent is allowed to run to waste until a positive ninhydrin colour is obtained. At this point, fractions are collected until the effluent is strongly alkaline. The amino-acid composition of the successive fractions is determined by paper chromatography (Consden, Gordon & Martin, 1944), whilst creatinine is identified by the Jaffé reaction as applied to paper chromatography (Maw, 1947). The degree of separation of the various ampholytes which is obtained by this primary fractionation is small owing to the large size of the columns. However, where interest is focused on one particular component, all the fractions which do not contain this substance may be discarded.

The secondary fractionation of the selected samples is carried out on a column system containing the anion-exchange resin Dowex 2. Some details on the use of this resin for the separation of amino-acid mixtures have been published (Partridge & Brimley, 1951*b*). The order in which the various amino-acids are displaced from this resin will be discussed later, but it can be stated here that the order is not simply the reverse of that obtained on the cation-exchange column. The ampholytes retained on the Dowex 2 columns are displaced with 0·1N-hydrochloric acid and again fractions are collected until the hydrochloric acid reaches the effluent. The fractions are analysed by means of paper chromatography as already described.

EXPERIMENTAL

Materials

The cation-exchange resin Zeo-Karb 215 (Permutit Company, London) was ground in a hammer mill and graded by sieving in the dried condition. The 40–60 mesh/in. grade was used for the large columns and 80–100 mesh/in. grade for small-scale use. The resin was allowed to stand overnight in 5N-HCl and, after several washes by decantation using distilled water, the columns were packed by sedimentation. The packed columns were treated once with N-NH_3 followed by water and then with 2N-HCl followed by water again until the pH of the effluent was 5. This alternate treatment with N-NH_3 and 2N-HCl served to consolidate the resin bed.

The anion-exchange resin Dowex 2 (Dow Chemical Company, Midland, Michigan), which is available in two grades, 30–40 and 250–500 mesh/in., and is supplied in the chloride form, was made up into a slurry with N-HCl and run into the columns. After allowing the resin to settle, it was washed with water and regenerated by applying 2N-NaOH (CO_2-free) followed by CO_2-free distilled water until the pH of the effluent fell below 8. Details of the precautions necessary to keep the system free from CO_2 have been reported (Davies, Hughes & Partridge, 1950).

Primary fractionation

In order to illustrate the method, a typical fractionation of human urine is described. The urine was collected from two subjects, both of whom excreted a proportion of β-amino-*iso*butyric acid which was high in comparison with the other amino-acids (Crumpler *et al.* 1951). The urine (18 l.) was evaporated to 2 l. under reduced pressure using a circulating evaporator (Van Heyningen, 1949), 3 vol. of ethanol were added and the mixture was allowed to stand overnight at 0°. After filtration, the solids were washed with 80% aqueous ethanol and the filtrate and washings were evaporated to remove the ethanol. The syrup was taken up with 3 l. of water and shaken with 100 g. of charcoal which had been previously treated with dilute acetic acid (Partridge, 1949). This procedure removed a large proportion of the tyrosine which tends to crystallize in the columns. Some phenylalanine is also retained on the charcoal. The clear filtrate obtained after removal of the charcoal was diluted to 36 l. and allowed to run on to the system of Zeo-Karb columns at a rate not exceeding 1 l./hr. The set of columns, already described (Partridge, 1950), was made of three sections placed side by side and connected up in series with narrow glass tubing. The first section (diam. 3 in., height 30 in.) contained 1100 g. (dry wt.) of Zeo-Karb 215 (40–60 mesh/in.); the second section (diam. 2 in., height 24 in.) contained

330 g. resin; and the final section (diam. 1·5 in., height 15 in.) contained 130 g. of resin. During the application of the solution, and also whilst a further 5 l. of distilled water was applied to replace the acid remaining in the columns, the effluent was allowed to run to waste.

The amino-acids were displaced by 0·2 N-NH_3 which was applied at a rate of 1 l./hr. Again the effluent was discarded until a positive ninhydrin reaction was obtained. At this point, fractions (50 ml.) were collected. Thirty-five fractions were taken including three collected beyond the point at which the dark NH_3 band reached the end of the last column. The amino-acid composition of the successive fractions was determined by placing 10 μl. samples at 2 cm. intervals along the base-line of sheets of no. 4 Whatman filter paper. One set was run with phenol-NH_3 and a duplicate set with collidine-lutidine mixture. After drying, the sheets were developed with ninhydrin in the usual way. Further sheets were prepared, run in the same solvents, and developed with alkaline picric acid solution to test the presence of creatinine.

with resin ground to about 60 mesh/in.; the third section (diam. 0·75 in., height 3 in.) again contained resin of 60 mesh/in.; whilst the final section (diam. 0·35 in. height 2·25 in.) was filled with resin of 250–500 mesh/in. The dimensions given are those for the internal diameter of the tubes and the height is the actual length of the column of resin. The tubes were, of course, longer to allow for head-space. It will be noticed that these columns are relatively shorter and wider than those used for the Zeo-Karb resin. This is due to the fact that the Dowex resin swells when the amino-acids solutions are applied and the use of long narrow columns would lead to the production of a considerable internal pressure. The fourth section, which restricts the flow rate through the system, was only placed in position just before the displaced amino-acids emerged from the third column, as shown by the swelling of the resin. The effluent, which contained creatinine, together with some creatine, was collected for investigation later. The columns were washed with 1 l. of distilled water after all of the

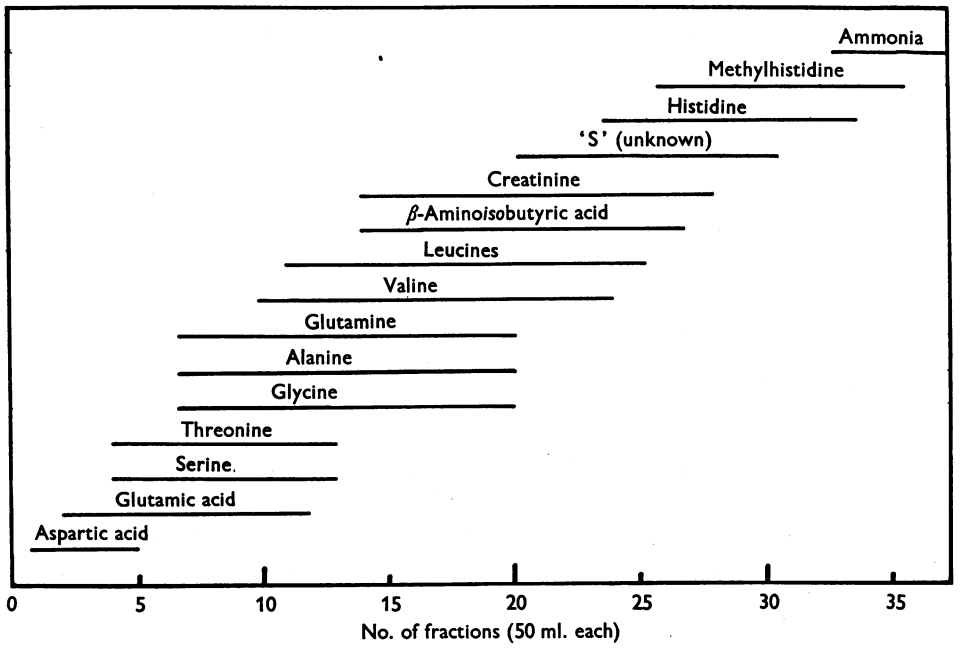

Fig. 1. Primary fractionation on Zeo-Karb 215.

Secondary fractionation

The first nine fractions from the previous separation which contained aspartic acid, glutamic acid, glycine and alanine, together with several other substances present in traces, were set aside. The remaining fractions (10–35), which contained the amino-acid selected for isolation, were bulked, heated to 60° under reduced pressure to remove CO_2 and applied to a system of columns containing the anion-exchange resin Dowex 2. This set was made up of four columns of decreasing size connected in series with narrow glass tubing. The first column (diam. 2 in., height 8 in.) contained the coarser grade of resin (approx. 30 mesh/in.); the second column (diam. 1·25 in., height 4·5 in.) was filled

amino-acid solution had run on. The amino-acids were now displaced by applying 0·1 N-HCl and fractions (25 ml.) were collected as soon as a positive ninhydrin reaction was obtained with the effluent. The collection was continued for two fractions beyond the point at which a positive chloride test was obtained. The amino-acid composition of the fractions was determined chromatographically in the manner already described.

RESULTS

The composition of the samples contained from the primary fractionation is shown in Fig. 1. It has been pointed out that considerable overlapping of the

amino-acid bands must be expected under the conditions imposed by the large quantity of inorganic salts present in the urine. A repeated fractionation using smaller columns, a reduced rate of flow during development and the collection of smaller and more numerous fractions will give a far greater resolution of the mixture. However, it is the writer's belief that it is preferable to proceed directly to a secondary fractionation on Dowex 2 and thus eliminate creatinine from the mixture. Then, if necessary, a further fractionation may be carried out on Zeo-Karb 215 of those selected fractions which contain the particular amino-acid which it is intended to isolate. Only those components which occur in relatively large amounts are indicated in Fig. 1. The composition of the fractions obtained from the

DISCUSSION

The method, so far outlined, is intended to provide an initial treatment whereby the various amino-acids and other ampholytes including simple peptides can be concentrated in a number of fractions. The actual isolation in pure form of any one component from this comparatively simple mixture will usually call for the use of additional methods, but the isolation of a substance occurring in large amounts relative to other amino-acids can often be achieved by ion-exchange fractionation alone. If, however, several amino-acids form mixed bands on both resins it may be possible to obtain a separation by using the Zeo-Karb column at a higher temperature and thus minimize the non-ionic

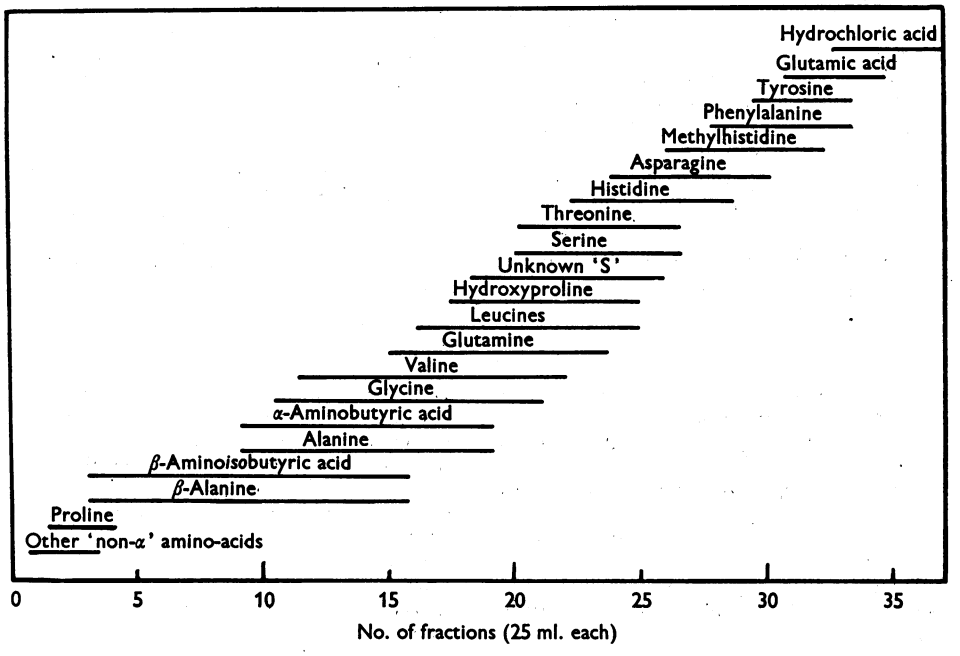

Fig. 2. Secondary fractionation on Dowex 2.

fractionation on Dowex 2 is shown in Fig. 2. The various amino-acids listed cannot all be recognized readily from one-way paper chromatograms, and it is necessary to carry out two-way chromatograms on doubtful fractions in order to obtain unambiguous identification. Also, with the exception of '*S*' substance, an unknown, the amino-acids named in the figure are limited to those which are the main constituents of urine (Dent, 1948). Consideration of other substances occurring in small quantities in these fractions will be reserved for a later paper.

absorption effect. Useful separations have been achieved in this way (Partridge & Brimley, 1951*a*). If the mixture is not resolved under these conditions it is unlikely that any separation will be gained by any method which depends on the electrical properties of the charged ampholytes, and other properties such as specific absorption, partition coefficients between aqueous and organic solvents or even purely chemical methods may have to be used.

Partridge & Brimley (1951*b*), in their paper on the behaviour of amino-acids on Dowex 2, indicated

the order in which these substances are displaced from the resin. This order is consistent with the theory (Davies, 1949) which assumes that the order of emergence is determined by the relative values of pK_2 of the amino-acids. Reference to the table of apparent dissociation constants of amino-acids (Cohn & Edsall, 1943) shows that apart from arginine and lysine, both of which are absent from the Dowex fractionation, those amino-acids in which the amino group is further removed from the carboxyl group than in α-amino-acids have values for pK_2 which are higher than those of α-amino-acids except proline and hydroxylysine. Consequently, it would be expected that these amino-acids would be the first to emerge from the Dowex 2 column, and reference to Fig. 2 shows that this is so. In fact it may be taken that any amino-acid, with the exception of proline and hydroxylysine, which is displaced from a Dowex 2 column under the conditions described in front of the alanine band is most probably a 'non-α' amino-acid. In the experiment described, the first three fractions from the Dowex column have yielded small amounts of several amino-acids in addition to β-alanine and β-amino*iso*butyric acid and proline. These unknown substances survive as visible spots on a paper chromatogram treated with basic copper carbonate (Crumpler & Dent, 1949). This is additional evidence that they are 'non-α' amino-acids.

SUMMARY

1. A procedure is described whereby the amino-acids and other ampholytes may be separated from other urine constituents and to some extent from each other.

2. The primary separation is carried out on columns of the cation-exchange resin Zeo-Karb 215. Selected fractions from the initial separation are then refractionated on columns of the anion-exchange resin Dowex 2. In this way, the ampholytes, as a group, are concentrated in a number of arbitary fractions and a large degree of resolution of the complex mixture is also obtained.

3. The selection of fractions in which any one amino-acid of special interest occurs provides a rich source for the final isolation of this substance.

4. The order in which the 'non-α' amino-acids are displaced from a column of the strongly basic resin Dowex 2 is discussed.

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REFERENCES

Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides.* New York: Reinhold.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.

Crumpler, H. R. & Dent, C. E. (1949). *Nature, Lond.*, **164**, 441.

Crumpler, H. R., Dent, C. E., Harris, H. & Westall, R. G. (1951). *Nature, Lond.*, **167**, 307.

Davies, C. W. (1949). *Biochem. J.* **45**, 38.

Davies, C. W., Hughes, R. B. & Partridge, S. M. (1950). *J. chem. Soc.* p. 2285.

Dent, C. E. (1947). *Biochem. J.* **41**, 240.

Dent, C. E. (1948). *Biochem. J.* **43**, 169.

Dent, C. E., Fowler, D. I. F. & Walshe, J. M. (1951). *Biochem. J.* **48**, xiii.

Maw, G. A. (1947). *Nature, Lond.*, **160**, 261.

Partridge, S. M. (1949). *Biochem. J.* **44**, 521.

Partridge, S. M. (1950). *Chem. & Ind.* **20**, 383.

Partridge, S. M. & Brimley, R. C. (1949). *Biochem. J.* **44**, 513.

Partridge, S. M. & Brimley, R. C. (1951*a*). *Biochem. J.* **48**, 313.

Partridge, S. M. & Brimley, R. C. (1951*b*). *Biochem. J.* **49**, 153.

Partridge, S. M. & Westall, R. G. (1949). *Biochem. J.* **44**, 418.

Searle, J. M. & Westall, R. G. (1951). *Biochem. J.* **48**, l.

Van Heyningen, W. E. (1949). *Brit. J. exp. Path.* **30**, 302.

Westall, R. G. (1950*a*). *Nature, Lond.*, **165**, 716.

Westall, R. G. (1950*b*). *J. Sci. Food. Agric.* **1**, 191.