SUMMARY

1. Muscle extracts of ten different species of fish have been examined by the paper-electrophoretic method in sodium phosphate buffer at $I=0.05$ and pH 7.55.

2. The recorded diagrams are similar in their main features, but differ in details characteristically from species to species. Thus it is possible to identify the fish by means of an electrophoretic analysis of the muscle extract obtained under prescribed conditions.

3. Pike muscle extract was analysed electrophoretically both on filter paper and in free solution. The diagrams obtained by both methods were found to run generally parallel.

4. Fractionation by precipitation with ethanol was applied to effect separation of the protein components extracted from pike muscle at low ionic strength. The proportions and the electrophoretic properties of protein components of different fractions were examined. The nature of the components of diagrams obtained before and after fractionation has been discussed.

The authors express their thanks to Professor E. Uroma, chief of the State Serum Institute, for permission to use the Tiselius electrophoretic apparatus and to Mr A. Louhivuori, Ph.M., for valuable assistance in conducting the electrophoretic analysis in free solution.

REFERENCES

- Connell, J. J. (1953*a*). *Biochem. J.* **54**, 119.
 Connell, J. J. (1953*b*). *Biochem. J.* **55**, 378.
 Dyer, W. J., French, H. V. & Snow, J. M. (1950). *J. Fish. Res. Bd Can.* **7**, 585.
 Hamoir, G. (1949). *Bull. Soc. Chim. biol., Paris*, **31**, 118.
 Hamoir, G. (1951). *Symp. biochem. Soc.* **6**, 8.
 Jacob, J. J. C. (1947). *Biochem. J.* **41**, 83.
 Kunkel, H. G. & Tiselius, A. (1952). *J. gen. Physiol.* **35**, 89.
 Mariani, A. (1952). *R.C. Ist. sup. Sanit.* **15**, 337.
 Reay, G. A. (1935). *Rep. Fd Invest. Bd, Lond.*, no. 65.
 Reay, G. A. & Kuchel, C. C. (1936). *Rep. Fd Invest. Bd, Lond.*, no. 93.
 Roth, E. (1947). *Biochem. Z.* **318**, 74.
 Smith, E. C. B. (1934). *J. Soc. chem. Ind., Lond.*, **53**, 351.
 Smith, E. C. B. (1937). *Proc. Roy. Soc. B*, **124**, 136.
 Subba Rao, G. N. (1948). Physico-chemical investigations on the muscle proteins of some commercial species of fish caught in N.E. Scottish waters. Ph.D. Thesis, University of Aberdeen.

The Amino Acids and other Ampholytes of Urine

3. UNIDENTIFIED SUBSTANCES EXCRETED IN NORMAL HUMAN URINE*

By R. G. WESTALL

Medical Unit, University College Hospital Medical School, London, W.C. 1

(Received 16 December 1954)

The application of the displacement method of chromatography on ion-exchange resins described in Part 1 of this series of papers (Westall, 1952*b*) has led to the isolation from urine of a number of previously unknown amino acids or ampholytes: β -aminoisobutyric acid (Crumpler, Dent, Harris & Westall, 1951), porphobilinogen (Westall, 1952*a*) and felinine (Westall, 1953). In the course of these studies certain other ninhydrin-reacting substances, many of which were unstable to acid hydrolysis, were observed but the amounts of these compounds were usually too small to permit further investigation. The existence of unknown substances in human urine which liberate free amino acids on acid treatment has been recognized for a long time (Henriques & Sørensen, 1909) and many other workers, notably more recently Boulanger, Biserte & Courtot (1952) and Carsten (1952), have added fresh evidence for the occurrence of such substances in urine. The elution diagram for normal urine (Stein, 1953) also showed a number of small peaks representing unknown ninhydrin-reacting substances, which disappeared after acid hydrolysis, in

addition to the larger peaks representing the commonly occurring amino acids. In this method, which uses only 2–5 ml. of urine, the analysis could not be expected to show substances excreted in amounts of less than 5 mg. per day, especially if they were peptides giving a relatively low colour yield with the ninhydrin reagent.

The work to be described now is an attempt to investigate further the ninhydrin-positive unidentified compounds in a sample of 100 l. of urine from a normal adult male subject. As a first method of isolation and fractionation of the ampholytes, ion-exchange chromatography has been employed using the displacement technique with its advantages of high capacity. This was followed by two-way paper chromatographic analysis of all the fractions obtained, with other methods of analysis as required.

EXPERIMENTAL

Urine (100 l.) was collected from one healthy, adult male subject over a period of 3 months. Only full 24 hr. specimens were taken and breaks were made over weekends owing to storage difficulties. The full collection represented the total output of 80 days. Each specimen was stored

* Part 2 of this series: Westall (1953).

at 0° and preserved with toluene and chloroform. The urine was worked up in four batches of 25 l., and each batch was fractionated as soon as sufficient urine was available.

The method used for the deproteinization of the urine and for the separation of the amino acids and other ampholytes by fractionation on the ion-exchange resins Zeo-Karb 215 and Dowex-2 has been described in detail in Part 1 of this series of papers (Westall, 1952b). The sizes of the columns of ion-exchange resins described therein were adequate to deal with the 25 l. of urine which had been partly desalted by the ethanol procedure. The amino acids and peptides were displaced from the primary column of Zeo-Karb 215 by 0.2N-NH₃ and the mixed effluent solution was applied directly to the secondary column of Dowex-2. 0.1N-HCl was used to displace the amino acids and ampholytes from this resin and the effluent was collected in 50 ml. fractions (30–32). These fractions were examined by paper-strip chromatography in order to blend together similar fractions from the four separate batches that were worked up. In this way 30 fractions each of 200 ml. were obtained representing the total ampholytes which had been held by both the ion-exchange systems.

Paper chromatography. Samples (100 μ l.) from the 30 fractions obtained were examined by two-way paper chromatograms using phenol-NH₃ and collidine-lutidine (Dent, 1948). An alternative system of phenol-NH₃ and butanol-acetic acid-water (40:10:5, by vol.) has also been used extensively. Paper chromatograms were also run after mixing 100 μ l. of the sample with 100 μ l. of conc. HCl and heating the mixture in a sealed tube (Pyrex glass) for 18 hr. at 105° and then removing the excess HCl under reduced pressure in a desiccator. A further series of chromatograms were run after oxidation of the sample with hydrogen peroxide and ammonium molybdate (Dent, 1948). Use has been made of the cutting-out technique (Consden, Gordon & Martin, 1947) whereby duplicate chromatograms were run and one developed with ninhydrin in the usual way. The second chromatogram was sprayed lightly with 0.025% ninhydrin (Sanger & Tuppy, 1951) near to but not actually on the spot to be cut out. In this way, by matching the two squares, the spot to be cut out for investigation could be accurately located without being contaminated with ninhydrin. After subjecting the substance extracted from the paper to the conditions of acid hydrolysis the product was run on small squares (Datta, Dent & Harris, 1950) using suitable markers. Although by using these smaller squares there is some loss in resolving power, there is also the added advantages of running up to twelve papers at a time and in the production of compact well-defined spots. With the small amount of substance derived from cutting out the original spot, the products produced by hydrolysis are often difficult to locate if re-run on a full sheet of paper (22½ × 18 in.). The specific spray reagents of Ehrlich, Pauly and Sakaguchi have been used extensively.

Paper electrophoresis. The method for separating the basic amino acids by ionophoresis on filter paper (Harris & Warren, 1954) has been employed as an additional means of identifying these substances. The separation of lysine and ornithine in particular is more readily achieved in this way than in the two-way chromatograms using the solvents described above.

Column chromatographic analysis. The chromatographic analytical technique of Moore & Stein (1951) has been used on selected fractions in order to plot the positions of certain peptides. The procedure has been carried out strictly

according to these authors' directions except that Dowex-50 resin of varying degrees of cross-linking has been used. Where reference is given to a particular run the degree of cross-linking of the resin is indicated. When it was necessary to check the identity of a peak on the flow diagram alternate fractions were combined and run on paper chromatograms. The desalting was carried out on columns (100 × 6 mm.) of Zeo-Karb 215 (100–200 mesh) in the H⁺ form. Up to 5 ml. of the 0.1M buffer solution could be safely handled on each column. After applying the solution for desalting the column was washed with 3 ml. of water and the amino acids displaced with 0.2N-NH₃. Since the quantity of amino acid on the column is very small it was necessary to collect only the last few ml. of effluent starting when the dark ammonia band reached within 1 cm. of the bottom of the column. The solution of recovered amino acid was then evaporated to dryness on a watch glass and adjusted to volume either directly for paper chromatography or for acid hydrolysis followed by paper chromatography.

Sub-fractionation on ion-exchange columns. In order to obtain fractions rich in various unknown amino acids or peptides with a view to their isolation, the original fractions (200 ml. each) were refractionated on small Zeo-Karb 215 and Dowex-2 columns, thereby increasing the resolving power of the system by taking a larger number of smaller fractions. The sizes of the columns varied with the quantities of amino acids to be handled but the general principles of the fractionations were essentially the same as those for the larger column separations.

RESULTS

A number of the ampholytes present in the original sample of urine are known to be eliminated in the course of the fractionation. Urea and taurine together with phenylacetylglutamine, which has been found to occur in urine in amounts of up to 500 mg./day (Stein, Paladini, Hirs & Moore, 1954), are not held by Zeo-Karb 215. Creatine, creatinine and arginine and the simple bases including ethanolamine are not retained by Dowex-2.

The order of displacement, from a column of Dowex-2 resin, of the commonly occurring amino acids (Partridge & Brimley, 1951) and other urinary constituents of a like nature (Westall, 1952b) has been established. Thus by later subjecting the fractions to two-way paper chromatography a degree of resolution was achieved which can, in a sense, be regarded as three-dimensional. This is particularly useful since it was often possible to distinguish substances which normally overlay each other on the paper chromatogram. For instance, in urinary chromatograms asparagine is usually blotted out by the glycine spot but after fractionation glycine occurred in fractions 3–16, whilst asparagine was found in 15–22, thus allowing unequivocal detection of each substance.

A diagrammatic picture of the substances found in the various fractions is given in Fig. 1. The horizontal lines represent the number of fractions in which each substance occurs; starting as a trace,

building up to maximum concentration and fading away to a trace amount. Since the mixture of amino acids and peptides was displaced from the Dowex-2 resin, which is unfunctional, with 0.1N-HCl, then the amounts of the substances present in each fraction should add up to a 0.1M concentration, assuming that each substance has only one acid group combining with the resin. In this way, by

The various unknown substances which have been detected on two-way paper chromatograms after spraying with ninhydrin have been plotted on a map in Fig. 2. A number of the common amino acids have also been included; this is because it is customary, in this laboratory, to allow the collidine-lutidine solvent to run off the end of the paper in order to obtain a greater spread of the amino acids, most of which have an R_f value of less than 0.5 in this solvent mixture. Hence it has been found preferable to recognize new spots by their relation to the pattern of the commonly occurring amino acids rather than by their R_f values. The unknown substances have been numbered and such information as is available about each one is given in the following section.

Unidentified ninhydrin-positive substances which are stable to acid hydrolysis

All references to paper chromatograms are those run in phenol-NH₃ and collidine-lutidine unless otherwise stated.

(1) This substance moved to a position just above proline and gave a typical ninhydrin purple colour. As it emerged so early in the fractionation it would be expected to be a fairly strong base or an ω -amino acid, but since it formed a copper complex (Crumpler & Dent, 1949) the latter possibility must be excluded. Pipecolic acid (Morrison, 1952) is known to move to this position on the chromatogram, but the unknown spot did not give the characteristic reddish fluorescence under ultraviolet light and the two substances behaved differently when chromatographed with butanol-acetic acid.

(2) Acid hydrolysis of substance (15) yielded a spot on the chromatogram which gave a purple colour with ninhydrin and was situated just below proline. This substance has not been found in the free state and its identity is unknown.

(3) Four substances are now known to occupy this position on the paper chromatogram: β -aminoisobutyric acid, methionine sulphoxide, sarcosine and this unknown substance. Methionine sulphoxide moves to the sulphone position after oxidation and could be eliminated on this evidence. Sub-fractionation of fractions (1-5) showed that the unknown and β -aminoisobutyric acid could be completely separated. However, the possibility remained that the unknown was sarcosine. The *p*-nitrobenzoyl chloride test on paper (Plattner & Nager, 1948) was negative, but Novellie & Schwartz (1954), who found sarcosine in the rock lobster, also found that this test failed with sarcosine. The elution curve for a normal urine (Stein, 1953) showed a small peak in the position assumed by sarcosine.

(4) A substance which gave a purple colour with ninhydrin was found in the position just below and slightly to the left of (3). It did not react to give

Fig. 1. The amino acid and ampholyte composition of the fractions obtained from the secondary fractionation on a column of Dowex-2 resin. The numbers refer to unidentified substances.

assessing the intensity of the ninhydrin spots on the paper chromatograms, it is possible to obtain an estimate of the amounts of each substance present. However, such assessments are only approximate, as certain ampholytes occur which do not give a colour with ninhydrin, and also there may be trace substances held by the resin by non-ionic adsorption.

a colour with the Ehrlich, Pauly or Sakaguchi reagents and the position of the spot was unchanged by oxidation.

(5) This substance gave a purple ninhydrin spot just to the right of valine. Methionine sulphone also takes up this position but this substance and the unknown have been shown to differ by chromatography in other solvents. The position of the spot is unchanged by oxidation.

having slightly lower R_F values both in phenol- NH_3 and in collidine-lutidine. This amino acid was also produced by substance (14) under the conditions of acid hydrolysis.

(7) This substance gave a brownish spot with ninhydrin, slightly below and to the right of aspartic acid. It was only seen after acid hydrolysis of fractions 19-21 and there is evidence that it arises from the degradation of 'Peach spot' (15). It

Fig. 2. A map of the positions taken up by the unknown ampholytes on the phenol- NH_3 and lutidine-collidine paper chromatogram. The sample is applied to the paper at the bottom right-hand corner. The numbers refer to the unknown substances (see text) and a number of known amino acids are included as reference spots. The contractions of Brand & Edsall (1947) are used.

(6) This substance moved to a position just above valine and gave an unusually vivid purple spot with ninhydrin. On oxidation it moved to the position (6A) and gave a brown ninhydrin colour. This substance is believed to be a new sulphur-containing amino acid. It was destroyed by Raney nickel, yielding only alanine. Hence it is probably a cysteine derivative and may be a lower analogue of feline (Westall, 1953) from which it differed by

also occurred after acid treatment of fraction (28) although 'Peach spot' was not present in this case.

(8) A purple spot occurred just over serine; it was only produced under the conditions of acid hydrolysis.

(9) A further purple spot occurred to the right of (8). This substance was also only produced after acid hydrolysis and was associated with the same fractions as (8).

(10) A reddish purple spot was found below glutamine. This substance did not occur free and was produced by acid hydrolysis.

Substances which gave a colour with ninhydrin but which are not stable to acid hydrolysis

(11) This substance gave a bluish purple spot just over lysine and produced an elongated shape similar to the behaviour of lysine itself. By cutting out the area of the spot and hydrolysing the substance eluted from the paper, arginine, lysine, histidine and glycine were obtained in approximately equal proportions (ninhydrin colour yield).

(12) A further elongated spot of bluish purple colour was found to the right of lysine in the position occupied by hydroxylysine. After cutting out and subsequent acid hydrolysis it yielded arginine, lysine, glycine and glutamic acid.

(13) This substance gave a weak bluish spot close to (12) which also occurred in the same fraction. However, by using butanol-acetic acid instead of collidine-lutidine as the second solvent the substance moved to a new position near valine from which it could be cut out without contamination. On acid hydrolysis it gave arginine and an unknown substance which gave a grey ninhydrin-coloured spot to the left of proline.

(14) This substance gave a bluish purple spot with ninhydrin just above the proline position. It was completely destroyed by acid hydrolysis and yielded lysine as the only ninhydrin-positive constituent. A refractionated sample rich in this compound was run on the Dowex-50 (12% cross-linked) analytical column (Moore & Stein, 1951). The unknown substance gave a peak between fractions 70-90, where serine and threonine occur when present in the mixture. The production of lysine was confirmed by hydrolysing the alternate fractions from the peak. From this evidence it would appear that the unknown is probably an acylated lysine compound but, as yet, the existence of a free organic acid, after hydrolysis, has not been demonstrated.

(15) A number of ninhydrin-reacting substances give spots in the 'under proline' position, and of these at least three occurred in fractions 1-5. Cutting-out experiments only gave confusing results, but the mixture was resolved by using the Moore & Stein column technique. This substance (15) gave a peak on the elution curve between 45 and 55 fractions after the buffer change from pH 3.41 to 4.25. This corresponds approximately to a position between valine and methionine on the standard curve with 8% cross-linked Dowex-50 (Moore & Stein, 1951) but in the present case a 4% cross-linked Dowex-50 was used and the relative positions may be slightly altered. The behaviour of this substance on paper chromatograms showed that

it differed from the following substance (16) in that it gave a delayed ninhydrin colour showing up slowly after about 12 hr. at room temperature and reaching a maximum intensity in 24-36 hr. Heating for 2 min. at 100° produced no colour and further heating only darkened the background. After acid hydrolysis substance (2) was produced giving a spot on paper almost in the same position as substance (15) but which gave a normal colour reaction with ninhydrin. Glutamic acid was also liberated in approximately equal amount.

(16) This substance was eluted from the Moore & Stein column by pH 4.25 buffer between 85 and 100 fractions after the buffer change from pH 3.41. This compound on paper gives with ninhydrin a purple colour which develops in the normal way (unlike 15). On acid hydrolysis it yielded approximately equivalent amounts of glutamic acid, glycine, lysine and an unknown base which moved to a position close to that of putrescine on the paper chromatogram. Paper ionophoresis experiments using pH 11.5 buffer (Harris & Warren, 1954) confirmed the presence of lysine and showed that the unknown base still had a net positive charge in this buffer system. There is no other evidence, so far, for the characterization of this unknown basic constituent, but a small quantity, 16 mg., of the parent substance has now been isolated.

(17) A purple ninhydrin spot occurred to the right of proline. After cutting out and subsequent acid hydrolysis it yielded glycine, lysine and β -alanine. There is still some doubt as to whether glycine is a true component since the fraction in which the substance occurs is rich in another substance, not hippuric acid, which fails to give a ninhydrin colour and which only gives glycine after acid treatment. However, the sub-fraction definitely contained no carnosine, anserine or pantothenic acid so that it is interesting to find another peptide which yields β -alanine after acid hydrolysis.

(18) A substance which gave an olive-green spot with ninhydrin occurred in the position between alanine and β -alanine. On the Moore & Stein column (Dowex-50, 4% cross-linked), it was eluted by pH 8.3 buffer just ahead of methylhistidine. After acid hydrolysis it yielded an unknown substance which gave a purple spot with ninhydrin on paper in a position to the left of leucine.

(19) A substance which gave a brown spot with ninhydrin occurred in a position well to the right of proline. After acid hydrolysis of the material obtained by cutting out the area of the spot on paper, about equal amounts of proline and hydroxyproline were obtained. The fraction in which this substance occurred contained no free proline or hydroxyproline, in fact hydroxyproline has not been found in the free state in any of the fractions obtained. There is a tendency for proline peptides to

form diketopiperazines (Smith, 1953), and Dakin (1920) actually isolated the prolinehydroxypropyl-diketopiperazine from acid-hydrolysed casein. He found, however, that concentrated HBr at 100° for 4 hr. was required to regenerate the component amino acids. Since it is unlikely that the diketopiperazine would give a colour with ninhydrin and also since this unknown substance was readily broken down with 5N-HCl, it must be assumed that the new compound is a true peptide. This assumption is also supported by its position on the paper chromatogram, moving further than hydroxyproline yet slower than proline in phenol-NH₃.

(20) This substance gave a bluish purple spot to the left of valine. Only glutamic acid was detected after acid hydrolysis. It is tentatively suggested that this substance may be theanine, the γ -ethylamide of glutamic acid which occurs abundantly in the tea-leaf (Sakato, 1950). The two substances behaved similarly on paper chromatograms using a number of different solvent systems. The subject who supplied the urine for the investigation usually consumes an average of 1.5 l. of tea each day.

(21) and (21A) This substance gave a greyish blue spot with ninhydrin and occurred in fractions 15–24. The position of the spot varied between the two places indicated on the map. When the substances eluted from the spots of varying position were subjected to acid hydrolysis, aspartic acid, glutamic acid and lysine were produced in each case. This may indicate that several peptides are present differing only in the relative amounts of the component amino acids, but it is also possible that this substance is particularly sensitive to the amount of NH₃, which is not rigorously controlled, in the chromatographic cabinet.

(22) This substance gave a purple spot just below tyrosine. It was destroyed by the conditions of acid hydrolysis and produced no substance which would give a ninhydrin spot on paper.

(23) In fractions 19–23 inclusive, histidine and another substance which gave a purple colour with ninhydrin were found to occupy the same position on the paper chromatogram. These two substances were completely separated by a series of fractionations on the smaller ion-exchange columns. This new substance was modified by heating at 105° with 5N-HCl and yielded substance (6). Moreover, on oxidizing the unhydrolysed substance a brown spot was again produced in position (6A). Also, as in the case of substance (6), alanine was detected after Raney nickel treatment. Apparently, therefore, these two substances are closely related but the nature of the relationship is unknown. Fifty mg. of this unknown (23) have now been isolated as a white powder but attempts to obtain a crystalline product have so far been unsuccessful.

(24) The occurrence of this substance in cat urine

has been reported (Westall, 1953), where it was designated as 'Peach spot' because of the characteristic colour it gave with ninhydrin. This spot has been seen in overloaded chromatograms from a number of urine samples from different human subjects and it is probably a commonly occurring excretory substance. It was unaffected by oxidation but was destroyed by heating with 5N-HCl at 105° and gave a brown ninhydrin spot in position 7. The substance has not been isolated, but in a previous experiment a fraction rich in this substance was passed through a column of Zeo-Karb 215 in the H⁺ form operated at 75°. Under these conditions it was found to be almost completely destroyed, giving an acid degradation product which was not held by the resin but which crystallized readily m.p. 154° (uncorr.), and contained no P, S, or halogen. However, it gave no colour with ninhydrin even after heating with acid, although the substance contained 20% N.

(25) This substance gave a purple ninhydrin spot below and to the right of histidine. After oxidation the original spot disappeared and a new brown spot (25A) occurred to the right of valine. The original substance is destroyed by acid hydrolysis and its fate is unknown. This is believed to be a sulphur-containing compound but it is not a cystine peptide.

(26) This substance gave a purple ninhydrin spot in the glutamine position and was destroyed by acid hydrolysis without yielding any ninhydrin-reacting products. In fact this substance behaved similarly to (25) and gave an oxidized spot close to (25A). However, they occurred in the same fractions giving separate spots and so must differ in other respects. Both this substance and (25) gave bleached spots on a darkened background with the platinum chloride (Winegard, Toennies & Block, 1948) and with the sodium azide spray reagents (Chargaff, Levine & Green, 1948), both of which detect sulphur compounds.

(27) A brown ninhydrin spot occurred between glutamic and aspartic acids. The position was unchanged after oxidation but the spot disappeared after acid hydrolysis. After cutting out and subsequent hydrolysis only glutamic acid and aspartic acid were found. It is therefore uncertain whether the unknown does in fact contain these two component amino acids or whether they arise owing to contamination.

(28) This substance gave a brown ninhydrin spot between tyrosine and tryptophan. It was unaltered by oxidation but disappeared after acid hydrolysis and the resulting product was undetectable with ninhydrin.

(29–38) These substances have been grouped together as little is known about them individually and they all occurred in the last four, i.e. the most acidic, fractions. They gave weak colours with

ninhydrin. However, after acid hydrolysis, they yielded relatively large amounts of aspartic and glutamic acids, glycine, alanine, valine, leucine(s), serine and threonine, together with lesser amounts of tyrosine, phenylalanine and proline and traces of β -alanine and β -aminoisobutyric acid. No arginine, lysine or histidine was found and the sulphur amino acids were also absent.

Other unknown substances

Those substances which gave coloured spots with ninhydrin and which occurred in more than one fraction have now been described. There are, however, still a number of substances present which did not react with ninhydrin to give a colour but which were detected by other means. For instance, it was possible to demonstrate the occurrence of eight substances which gave a colour with the Pauly reagent. Of these, excluding histidine, carnosine and tyrosine, four were stable to acid hydrolysis. One of these substances appeared to coincide with urocanic acid, already found in human urine (Inoue, 1953) and the others are probably other imidazole derivatives. By viewing the paper chromatograms under an ultraviolet lamp (Osira, General Electric Co., Kingsway, W.C. 2), at least four well-defined fluorescent spots were recorded; one was just above phenylalanine, another above and to the left of tyrosine, whilst the two others were near the glycine position but differed in colour, one giving a very intense blue shade.

The anomalous behaviour of asparagine when oxidized with H_2O_2

In the course of running oxidized two-way chromatograms on the series of fractions it was noticed in fractions 15–22 that free aspartic acid was produced, whereas the unoxidized chromatograms showed no aspartic acid. The origin of this substance remained unknown until the source was finally traced to asparagine. As far as the author is aware it is not generally known that when asparagine is subjected to the conditions used for oxidation (Dent, 1948) about one-third breaks down to produce aspartic acid. Glutamine, which is regarded as the more unstable amide, yields no glutamic acid under these conditions and the intensity of the ninhydrin colour of the glutamine spot is not appreciably diminished.

DISCUSSION

The present analysis of 100 l. of urine from one normal male subject has shown clear evidence of the presence of thirty-eight unidentified ninhydrin-reacting substances (of which ten are acid stable). These were in addition to the twenty-seven known substances which were identified chromatographic-

ally, most of them well known as urinary constituents. The unidentified substances are presumably excreted in the urine of this subject in amounts of less than 10 mg./day or they would all have been detected by earlier urine analyses using the Moore & Stein technique of ion-exchange chromatography (Stein, 1953). In fact, a specimen of urine from the same subject was analysed in this laboratory using Stein's method and showed only seven small peaks due to unidentified compounds, the elution diagram resembling closely those published by Stein (1953) for normal urine (Dr D. F. Evered, private communication). Nor have we encountered such large numbers of unknowns on the overloaded (sample containing 1.25 mg. N) two-way paper chromatograms frequently carried out in this laboratory. In contrast, the displacement chromatographic method described here, when applied to a sample of 100 l. of urine, has enabled substances to be detected when excreted in quantities of only 0.5–1.0 mg./day.

The loss, in this fractionation, of a number of well-known urinary constituents was expected and has already been explained. There are still others we expected to find but whose presence has not been demonstrated. Since lysine is not fully recovered from the Zeo-Karb 215 columns when displaced with 0.15N- NH_3 , this may also apply to ornithine, which was found only in unexpectedly small amounts. A further small amount of ornithine was liberated by acid hydrolysis but the source is still unknown. Glutathione was not found but it may have been present in low concentration in those fractions (20–25) which contained cystine and which yielded glycine and glutamic acid after acid hydrolysis. The presence of α -aminoadipic acid has not been shown but it moves to the same position as glutamic acid on the two-way chromatogram and would be expected to occur in the same fractions.

Of the other less well known urinary constituents, carnosine and anserine were found in amounts approximately equivalent to an excretion of 2–3 and 5–7 mg./day respectively. Free 1-methylhistidine occurred abundantly and the recently found 3-methylhistidine (Tallan, Stein & Moore, 1954) was present almost to the same extent. There was no evidence for the occurrence of a peptide similar to anserine but involving 3-methylhistidine instead of the 1- isomer. β -Alanine was found free and a considerably greater amount was released by acid hydrolysis, more, in fact, than could be accounted for by carnosine, anserine or the new peptide (17).

The occurrence of a peptide containing only proline and hydroxyproline is of considerable interest. It is reasonable to assume that this substance is derived from collagen degradation but work on the systematic breakdown of this protein under mild hydrolytic conditions has not, as yet,

produced any peptides containing proline linked directly to hydroxyproline (Schroeder, 1953). The existence of a basic peptide (11) containing arginine, lysine, histidine and glycine is also of interest. It, too, may represent a product of protein degradation, since Acher, Thaureaux, Crocker, Justisz & Fromageot (1952) isolated arginylhistidine and arginylhistidyllysine from a controlled hydrolysis of lysozyme.

Whilst it is well known that urine contains a relatively large amount of bound α -amino nitrogen, blood plasma is comparatively free from these conjugates (Stein & Moore, 1954). Presumably therefore these conjugates or peptides, unlike the simple amino acids, are not appreciably reabsorbed by the kidney tubule from the glomerular filtrate. Christensen, Lynch & Powers (1946) have also produced evidence to support such differential handling by the kidney tubule.

When we consider the presence of peptides in body fluids three possibilities come to mind: they may represent specific fragments required for protein synthesis; they may arise from dietary protein degradation, representing products absorbed into the blood stream but unsuitable for protein synthesis; they may represent, in some cases, hormones or other physiologically active substances. This last possibility more easily explains their escape into the extracellular fluids and urine, and conforms well with the recent findings that certain hormones such as oxytocin and ACTH are indeed small-molecular-weight peptides.

With regard to the fractionation as a whole, the peptides which were displaced from the Dowex-2 column in the early fractions contained a preponderance of the basic amino acids and appear to be comparatively simple substances containing at the most four different amino acids although, of course, this does not necessarily imply a low molecular weight. As the fractionation progressed the number of different amino acids present in the peptides increased until in the most acidic fractions they yielded most of the neutral and acidic amino acids but contained no arginine, lysine or histidine. Boulanger & Biserte (1953) likewise found that the peptides in the acidic fractions from an anion-exchange fractionation of a pepsin hydrolysate of crystalbumen yielded only traces of the basic amino acids. Synge (1953) remarked on the frequent occurrence of comparatively simple peptides which contain the neutral and acidic amino acids only and considered that they might well be important intermediate protein metabolites. It may be that in the course of protein synthesis such peptides are linked together by peptides containing only the basic amino acids such as have been found in this urine fractionation.

The occurrence in urine of a large number of

unknown substances which give coloured spots with ninhydrin on paper chromatograms and which occupy positions on the paper near to and, in some cases, actually coinciding with those of the well known commonly occurring amino acids emphasizes the care that is required when interpreting chromatograms of biological fluids.

The amounts of many of the unknown substances found during the course of the fractionation of 100 l. of normal urine, is too small and the mixture still too complex to hope to isolate, in a pure state, more than a few of them. Increasing the amount of urine by 10 times would yield larger amounts of these substances but it might also, by increasing the sensitivity of the method, reveal an ever greater complexity. Indeed there were many further substances detected in this fractionation which have been omitted from consideration since they only appeared in traces in 1-2 fractions and could not be properly investigated. There is, however, always the possibility that some of these unknowns may occur in larger amounts in some pathological urines or in other biological fluids or tissue extracts which would thus provide a better source for their final isolation and identification. It is hoped that the chromatographic map of these unknowns which has been compiled will assist in making comparisons between unidentified substances from various sources. It would seem that some revision of our present concepts of amino acid metabolism may be required following identification of these numerous new metabolites.

SUMMARY

1. The amino acids and other ampholytes from 100 l. of urine from a normal adult male subject have been studied by fractionation on ion-exchange columns followed by chemical and paper chromatographic procedures.

2. The order of their displacement from Dowex-2 resin is recorded.

3. Twenty-seven of the ninhydrin-reacting substances could be identified by paper chromatography. A further thirty-eight substances, five of which appeared after hydrolysis, gave spots not corresponding to known substances. These have been plotted on a chromatographic map.

4. Ten of the unknowns are stable to the conditions of acid hydrolysis.

5. The remaining twenty-eight substances are unstable to hydrolysis, mostly yielding varying numbers of the common amino acids.

The author wishes to express his gratitude to Dr C. E. Dent for his interest and encouragement during the course of this work. He also wishes to thank Miss Valerie Arrow for carrying out the amino acid analyses by the Moore & Stein chromatographic method.

REFERENCES

- Acher, R., Thaureaux, J., Crocker, C., Jutisz, M. & Fromageot, C. (1952). *Biochim. biophys. Acta*, **9**, 339.
- Boulanger, P. & Biserte, A. (1953). *The Chemical Structure of Proteins*, p. 165. Eds. Wolstenholme & Cameron. London: Churchill.
- Boulanger, P., Biserte, G. & Courtot, F. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 621.
- Brand, E. & Edsall, J. T. (1947). *Annu. Rev. Biochem.* **16**, 224.
- Carsten, M. E. (1952). *J. Amer. chem. Soc.* **74**, 5954.
- Chargaff, E., Levine, C. & Green, C. (1948). *J. biol. Chem.* **175**, 67.
- Christensen, H. N., Lynch, E. L. & Powers, J. H. (1946). *J. biol. Chem.* **166**, 649.
- Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
- 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.
- Dakin, H. D. (1920). *J. biol. Chem.* **44**, 499.
- Datta, S. P., Dent, C. E. & Harris, H. (1950). *Science*, **112**, 621.
- Dent, C. E. (1948). *Biochem. J.* **43**, 169.
- Harris, H. & Warren, F. L. (1954). *Biochem. J.* **57**, xxxii.
- Henriques, V. & Sørensen, S. P. L. (1909). *Hoppe-Seyl. Z.* **63**, 27.
- Inoue, M. (1953). *Nagasaki Iggakki Zasshi*, **28**, 1283.
- Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
- Morrison, R. I. (1952). *Biochem. J.* **50**, xiv.
- Novellie, L. & Schwartz, H. M. (1954). *Nature, Lond.*, **173**, 450.
- Partridge, S. M. & Brimley, R. C. (1951). *Biochem. J.* **49**, 153.
- Plattner, A. & Nager, U. (1948). *Helv. chim. acta*, **31**, 2203.
- Sakato, Y. (1950). *J. agric. chem. Soc. Japan*, **23**, 262.
- Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 463.
- Schroeder, W. A. (1953). *The Chemical Structure of Proteins*, p. 184. Eds. Wolstenholme & Cameron. London: Churchill.
- Smith, E. (1953). *The Chemical Structure of Proteins*, p. 194. Eds. Wolstenholme & Cameron. London: Churchill.
- Stein, W. H. (1953). *J. biol. Chem.* **201**, 45.
- Stein, W. H. & Moore, S. (1954). *J. biol. Chem.* **211**, 915.
- Stein, W. H., Paladini, A. C., Hirs, C. H. W. & Moore, S. (1954). *J. Amer. chem. Soc.* **76**, 2848.
- Synge, R. L. M. (1953). *The Chemical Structure of Proteins*, p. 50. Eds. Wolstenholme & Cameron. London: Churchill.
- Tallan, H. H., Stein, W. H. & Moore, S. (1954). *J. biol. Chem.* **206**, 825.
- Westall, R. G. (1952a). *Nature, Lond.*, **170**, 614.
- Westall, R. G. (1952b). *Biochem. J.* **52**, 638.
- Westall, R. G. (1953). *Biochem. J.* **55**, 244.
- Winegard, H. M., Toennies, G. & Block, R. J. (1948). *Science*, **108**, 506.

Biochemistry of the Wood-rotting Fungi

8. VOLATILE METABOLIC PRODUCTS OF *DAEDALEA JUNIPERINA* MURR.*

BY J. H. BIRKINSHAW AND P. CHAPLEN

Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

(Received 20 November 1954)

A sample of East African pencil cedar, *Juniperus procera* Hochst., which showed a crumbling brown rot in the heartwood, was received in 1937 at the Forest Products Research Laboratory and a fungus was isolated but could not at first be identified. However, some years afterwards a culture of *Daedalea juniperina* Murr. isolated by Dr R. W. Davidson of the U.S. Department of Agriculture in Washington was obtained and this proved to be identical with the fungus isolated from the decayed pencil cedar. An unusual rather sickly sweet aromatic smell was produced in these cultures. Through the courtesy of Dr W. P. K. Findlay, who supplied the cultures, we were able to investigate the metabolic products of the fungus in continuation of previous work on the odorous constituents of wood-rotting fungi (cf. Birkinshaw, Morgan & Findlay, 1952).

* Paper 7 of this series: Birkinshaw, Morgan & Findlay (1952).

Daedalea juniperina is widely distributed but not common in the eastern and southern parts of the U.S.A. on living trees, worked timber, and stumps of *Juniperus virginiana* and has been reported on *J. excelsa* from Russia. The sporophore, which is a thick greyish corky bracket with a daedaloid pore surface, is generally similar in appearance to *Daedalea quercina*, which is so common on oak.

The fungus was grown mainly on a synthetic medium containing glucose and inorganic salts together with a little Marmite to supply vitamin B₁. At harvesting, the whole contents of the culture flasks was subjected to steam distillation, and the volatile products thus separated were examined and form the subject of the present communication. Other metabolic products present in the culture fluid and in the mycelium are still under investigation. The steam distillates on extraction with ether afforded a small amount of oil from which, by fractionation with light petroleum, colourless