A Study of the Behaviour of some Sixty Amino-acids and other Ninhydrin-reacting Substances on Phenol-'collidine' Filter-paper Chromatograms, with Notes as to the Occurrence of some of them in Biological Fluids

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Although the method of filter-paper chromatography (Consden, Gordon & Martin, 1944) can be applied to very many other types of compound (Partridge, 1946; Dent, 1947*a*; Vischer & Chargaff, 1947; Lugg & Overell, 1947; Maw, 1947), it is likely that its value will continue to be greatest in the amino-acid field. In this paper is presented a 'map of the spots' which may be of help to other workers. All the common amino-acids and many rare ones have been studied, also a few other interesting substances, some synthetic and not likely to be encountered in nature, which also give a ninhydrin colour reaction resembling that given by α -amino-acids. Some of the commoner 'unknowns' are also included since they appear to represent new compounds of wide distribution. To include all the 'unknowns' would be difficult, since it has been usual, when working with substances from natural sources, to stumble on them when least expected and in the most bewildering profusion.

EXPERIMENTAL

Points are only raised here which are additional to the earlier recorded work (Consden *et al.* 1944; Dent, 1946, 1947*a*).

Apparatus. The cabinet is shown in Fig. 1. It is convenient to use and construct. A cabinet of this type has given satisfactory service for two years of almost continuous use. It is much better to have two cabinets, one for the phenol and the other for the collidine runs.

Solvents. The phenol must be of A.R. or B.P. quality; too much decomposition with the formation of dirty brown material occurs if cruder phenol is used. An occasional batch of 'pure' phenol has been encountered which has decomposed unduly in spite of all possible precautions. It is a waste of time to persevere with such a solvent.

The nature of the collidine used has been of particular importance. Pure 2:4:6-collidine (2:4:6-trimethylpyridine) obtained from two different American sources completely failed to reproduce the results obtained in England by Consden *et al.* (1944) and by the writer, who all used the '2:4:6-' compound obtained from a British source. The R_F values were at least half of their reported ones, the insufficient separation of the amino-acids which thus ensued making the finished chromatograms almost worthless. On the other hand, a crude American 'collidine' believed to contain very little of the 2:4:6-isomer gave excellent results. By the

kindness of the Reilly Tar and Chemical Corporation samples of pure 2:3-, 2:4- and 2:5-lutidines, of 2:4:6- and 2:3:6-collidines, of aldehyde-collidine (2-methyl-5-ethylpyridine) and of some crude boiling fractions of pyridine homologues were provided and tried out. The original British results could be well reproduced by taking a mixture of 1 vol. of

Fig. 1. Diagram of cabinet used in two-dimensional chromatography. The cabinet (A) is best made of $\frac{3}{4}$ in. thick 5-ply wood with the sides of one piece. The inside dimensions are $32 \times 32 \times 8$ in. It is provided with a glass window 24×24 in. on each side. It must be screwed together firmly owing to a tendency to warp. The inside of the wood is heavily painted with paraffin wax. It is provided inside with a shelf (D) at both ends placed 4 in. from the top, the central notch takes the long glass trough, the lateral ones are used as alternative positions for the glass rods. The under-surface of the lid (B) has a rubber gasket which makes an airtight fit against the top edge of the cabinet when screwed down by the wing nuts as in C.

2:4-lutidine and of 1 vol. of 2:4:6-collidine and saturating it at the working temperature with 2 vol. of water. This mixture has been used here and is referred to in future as 'collidine'. This is not claimed to be the best possible mixture, and there is scope for further improvement to meet special points. When pure isomers are not available a crude fraction of pyridine bases boiling around 160° may be adequate. If the R_F values are too large and the spots ill-defined in consequence the fraction can be mixed with more

or less of the fraction boiling around 170°. There is some relation between the capacity of the solvent to take up water and its suitability for paper chromatography of amino-acids. When 5 ml. of water and 5 ml. of a good 'collidine' are mixed at about 23°, only about 1 ml. of the water remains undissolved. So far, when the water left over measures more than 1.5 ml. the solvent has given uselessly low R_F values.

General methods

A variation of more than 5° in temperature during a run is believed to be harmful, at least during the collidine run. The cabinet is therefore always kept, if possible, in an inside room without windows. The operating temperature is, however, less critical than was previously thought. Excellent results have been obtained at room temperatures of 12–31°.

The phenol was always used as the first solvent. Addition of about 0.1 g. of NaCN to one of the beakers in the bottom of the box was a great help in preventing oxidation of phenol. Just before closing the cabinet at the beginning of a run, four drops of ammonia solution (sp.gr. 0.880) were dropped in down the side. Four drops of diethylamine (Consden, Gordon & Martin, 1947) were likewise added just before a 'collidine' run. The sheets were dried at room temperature by directing an electric fan on to them overnight. The ninhydrin reaction was allowed to take place during 24 hr. at room temperature (24–30°). This avoids the danger of fading of the colour owing to overheating in the oven. With the exception of α -amino-isobutyric and of α -amino- α -methylbutyric acids (*q.v.*) all the amino-acids readily develop in this way. No difficulties with the 'pink front' of Consden *et al.* (1944) due to contamination by Cu salts have ever been encountered, even when no cyanide was added to the cabinet. Occasional batches of ninhydrin have been purchased that fail completely to give any colours with amino-acids.

The amino-acids were normally used in the form of a 0.01 M-solution, 5–10 μ l. being nearly always adequate to give a good spot on the paper. The standard solutions are stable over many months if made up in 75% (v/v) ethanol, a small amount of conc. HCl being added to dissolve the less soluble ones. When reference is made to the appearance of this or that amino-acid in urine or blood it is to be assumed that volumes of 25 μ l. of urine and of 125 μ l. of protein-free plasma have been taken for the chromatogram (see Dent, 1946). There has been no systematic attempt to determine the lower limit of sensitivity for the detection of each amino-acid; the figures quoted are therefore usually on the high side. No mention is made as to the optical species, since there is good reason to believe that the active compounds and racemic mixtures travel similarly on the paper. The numerous substances which give very weak colours with ninhydrin are not included in the map, since under the conditions imposed, they are not likely to be present in large enough amounts to be confused with simple amines.

R_F values are not mentioned as such any longer. The rough figures can be surmised from Fig. 2 which is drawn to scale. The values in 'collidine' vary so widely with the isomer composition as to be worthless to record. Values in phenol are much more constant, but there is an occasional unaccountable variation of up to 15%, except under carefully controlled conditions, as when two sheets are run simultaneously in the same cabinet. It is stressed that the safest and simplest method of identification, now used exclusively,

is from the pattern of the spots. This is remarkably constant and can be seen at a glance when many amino-acids are present together. When only a few are present the most convenient amino-acids to add as markers are glycine, alanine, valine and leucine. This extra step is not usually necessary because these same amino-acids are the ones most commonly present in greatest amounts in biological fluids.

Special methods

Effect of acidity during the phenol run. The basic amino-acids travel much more slowly under conditions of acidity which have little if any effect on the monoaminomonocarboxylic acids (Consden *et al.* 1944). This is, indeed, a very good test for basicity. The effect is even more marked with more basic substances such as glucosamine (*q.v.*). Perhaps the best way of carrying this out is to introduce during the phenol run a beaker of 50% (v/v) acetic acid into the cabinet, and, of course, to omit the ammonia. Equal volumes of conc. HCl and water may also be used in the beaker, but sometimes this mixture will also slow considerably the monoaminomonocarboxylic acids. The writer cannot confirm the claim of Consden *et al.* (1944) that the monoaminodicarboxylic acids travel quicker in phenol under acid conditions. Anomalies of their behaviour are discussed further under 'glutamic acid' and 'aspartic acid' in the section on results.

Hydrogen peroxide treatment. This continues to be a valuable technique for investigating the sulphur-containing amino-acids. The H_2O_2 is applied on the paper itself as previously described (Dent, 1947a), or about 5 μ l. may be mixed beforehand with the sample. An interesting point, also discussed later, has arisen with regard to methionine sulphoxide. This substance does not oxidize further to the sulphone under the usual conditions which are adequate to oxidize methionine completely. Indeed, as far as can be judged from the chromatographic method, when a mixture of methionine and methionine sulphoxide is treated with H_2O_2 the methionine is converted to the sulphone, this presumably having to go through the sulphoxide stage of oxidation, while the sulphoxide which was present from the start remains largely unchanged. Addition, however, of 10–20 μ l. of 0.02% ammonium molybdate to the spot on the paper allows the H_2O_2 to convert both methionine and its sulphoxide to the sulphone. There was always increased decomposition of the phenol after H_2O_2 had been used on the paper.

Routine method of investigating a potentially interesting fluid

The following routine is almost unavoidable with a new mixture containing many amino-acids, if all of them are to be detected and identified:

- (1) Strip method (one-dimensional chromatogram) run with phenol. This serves as a rough qualitative and quantitative test. The volume of solution taken for the further tests may have to be adjusted on the basis of this result.
- (2) Strip method with 'collidine'. Development is effected with the Pauly diazo reagent (Pauly, 1904) to show histidine and other diazo-reacting substances, which may not appear in the later tests owing to their relatively weak ninhydrin colour reactions.
- (3) Paper square (two-dimensional) run with phenol and 'collidine'. This detects nearly all the amino-acids.

(4) Same as (3), but with prior H_2O_2 treatment. This detects cystine and methionine and will also affect other spots if due to divalent sulphur compounds (*q.v.*).

Further chromatograms may be necessary to cover specific points, especially after additions of pure amino-acids to act as markers.

Fig. 2. The 'Map of the Spots'. The crosses show the average positions taken up on phenol-'collidine' two-dimensional chromatograms by the substances in question. See text for the identifications. The map is drawn to scale, assuming that the amino-acids start from the point at the right-hand bottom corner, and that the phenol is allowed to soak first along the paper from right to left, as far as the extreme left edge, then the collidine in an upward direction as far as the top edge. Approximate R_F values can be deduced from the scales at the edges. In practice, owing to the lower average R_F values in 'collidine', it is more convenient to run the 'collidine' for a greater distance on the paper than the phenol. This has the effect of spreading the spots upwards to a proportionate extent.

(5) Same as (3), but after hydrolysis with 6N-HCl for 24 hr. at 100°. The HCl must be removed by evaporation and the solution then made up to its original volume. Spots due to peptides, amides or tryptophan are removed by this process. Amino-acids liberated by the hydrolysis appear as new spots or as a strengthening of the spots already given by the unhydrolyzed fluid. It is important to remove traces of protein before the acid hydrolysis.

RESULTS

The behaviour of each amino-acid can be seen from the map (Fig. 2) and from the summaries below. Substances marked with an asterisk have only been run a few times on the chromatograms, and the results should be accepted with more reserve than

those from the other amino-acids, many of which have been run hundreds of times and under very variable conditions. The position of each substance on the map is shown by the number in parentheses after the name. The colour mentioned refers to the ninhydrin reaction and is to a minor extent dependent on the composition of the 'collidine' used.

Amino-acids or other known substances shown on the map

α-Alanine (20). Detectable in amounts of 2 μ g. Gives a purple colour. Nearly always found in urine and other body fluids.

β-Alanine (29). Detectable in amounts of 5 μ g. Gives a bluish purple colour. Its position on the map is so close to that occupied by citrulline that there is a serious possibility of confusion, indeed simple position matching has so far always failed to make distinction possible. See 'Citrulline' for a useful spot test. A substance giving a colour in this position is often found in blood, urine and in plant and tissue extracts. It has been proved to be *β*-alanine in some of these cases.

**alloThreonine* (15). Detectable in amounts of 6 μ g. Gives a purple colour. This unnatural amino-acid cannot be distinguished from threonine either in these solvents or in butanol.

α-Amino-n-butyric acid (26). Detectable in amounts of 4 μ g. Gives a purple colour. This substance has been found in nearly every plant or animal tissue extract so far examined. It is usually present in much smaller amount than its neighbours, alanine and valine. It has not been found in acid hydrolysates of natural proteins, although it has been seen occasionally in the hydrolysates of chemically modified proteins, or in alkaline hydrolysates. Actual isolation in bulk for final confirmation of its structure remains to be done.

**α-Aminoisobutyric acid* (37). Detectable in amounts of 4 μ g. Gives a purple colour. The ninhydrin colour reaction occurs much less readily than with *α*-amino-*n*-butyric acid and the other common *α*-amino-acids. No colour develops after several days at room temperature. It soon appears, however, on heating to 100°. This is presumably due to a steric effect from the fully substituted *α*-carbon atom (see also *α*-Amino-*α*-methylbutyric acid). The unusual behaviour with ninhydrin allows of an easy distinction from the isomeric *n*-compound. This acid has not yet been found in nature.

γ-Aminobutyric acid (40). Detectable in amounts of 5 μ g. Gives a purple colour. A substance has been found in traces in blood and urine which matches its movements exactly. Both are stable to H_2O_2 , to prolonged acid hydrolysis, and are not affected by running the chromatogram at an acid pH during the phenol run. It is probably one of the substances found in fair quantity by Dent, Stepka & Steward

(1947) in potato extracts (no. 23 in their photograph). In the absence of close neighbours to act as reference points it may be confused with the commonly occurring methionine sulphoxide. In such a case, its identity may be confirmed by adding markers or by its stability to H_2O_2 in the presence of ammonium molybdate.

**ε-Aminohexanoic acid* (55) (*ε*-amino-*n*-caproic acid). Detectable in amounts of 5 μ g. Gives a purple colour. The ninhydrin reaction occurs as readily as with the *α*-amino-acids. Its position is very different from that occupied by its isomer, norleucine. The acid is not believed to occur in nature.

**α-Amino-ε-hydroxycaproic acid* (38). Detectable in amounts of 10 μ g. Gives a purple colour. Not believed to occur in nature. A spot in this exact position has, however, been given by the acid hydrolysate of deaminized casein, i.e. casein treated with HNO_2 . In the same hydrolysate only a trace of lysine was found.

**α-Aminooctanoic acid* (43) (*α*-aminocaprylic acid). Detectable in amounts of 6 μ g. Gives a purple colour. It has not yet been found in nature.

**δ-Aminopentanoic acid* (57) (*δ*-amino-*n*-valeric acid). Detectable in amounts of 5 μ g. Gives a purple colour. The ninhydrin reaction occurs as readily as with the *α*-amino-acids. Its position is very different from that occupied by its isomer norvaline. The acid is not believed to occur in nature.

**α-Aminophenylacetic acid* (33). Detectable in amounts of 8 μ g. Gives a purple colour. It is not believed to occur in nature.

Arginine (56). Detectable in amounts of 15 μ g. Gives a purple colour. In the presence of 1:1 (v/v) acetic acid it takes up the position 56A. The rate of movement in phenol is slowed still further in the presence of 5*N*-HCl. Its presence may be confirmed by means of the Sakaguchi reaction directly applied to the spot on the paper (Consden, Gordon & Martin, 1946a). However, this reaction is of limited value since it is less sensitive than the ninhydrin test and does not work well on spots previously developed with ninhydrin. Arginine is very commonly found in biological fluids.

Asparagine (13). Detectable in amounts of 5 μ g. Gives an orange brown colour, sometimes tinged with olive. Although it moves very close to glycine, overlapping always taking place when both are present, it can usually be distinguished by the difference in the colour. Presumably a small amount of asparagine could not be detected in the presence of a large amount of glycine, since the large spot then given by the latter would completely overlap the former. Asparagine has moved rarely to a position slightly above glycine. It has been found in pathological urine, and, of course, in large amounts in plant extracts and also in Amigen—a commercial brand of enzymic casein hydrolysate. It must not be

confused with an unidentified substance, stable to hydrolysis, which also gives a yellow spot and is present in biological extracts. This substance (not shown on the map) moves to a position just below asparagine.

Aspartic acid (5). Detectable in amounts of 5 μ g. Gives a bluish purple colour which develops a little more slowly at room temperature than do the colours of the other common amino-acids. The spot usually shows streaking in the direction of the line shown, or may even appear as two distinct spots one over the other. The reason for this is not yet known. The acid is not usually found in normal urine, but often in pathological ones with a high concentration of amino-acids (Dent, 1947b). When found in natural sources the spot is often distorted by an unidentified yellow substance which moves between aspartic and glutamic acids.

Urines which do not contain detectable aspartic acid or asparagine often contain large amounts of the former after acid hydrolysis. Presumably the aspartic acid is present in the original urine as a simple derivative, such as an acyl one.

**Carnosine* (54). Detectable in amounts of 15 μ g. Gives a brownish yellow colour which turns greenish if heated to 105°. It has not yet been found in blood or urine.

Citrulline (30). Detectable in amounts of 5 μ g. Gives a reddish purple colour. It may be confused with β -alanine which moves to a similar position. It can be distinguished from β -alanine by treating the spot on the paper with 5% (w/v) *p*-dimethylaminobenzaldehyde in *N*-HCl with which it gives a bright yellow colour, in common with urea and many other urea derivatives, whereas β -alanine does not give a colour reaction. Its previous treatment with ninhydrin does not interfere. The aldehyde reaction for citrulline is less sensitive than the ninhydrin colour reaction, hence a negative test must only be considered significant when the ninhydrin reaction is very strong. A weak spot in the position of citrulline and giving the typical reddish purple ninhydrin colour is often seen in normal blood and urine. However, the presence of citrulline in these fluids has not yet been confirmed by the spot test.

Cystathionine (7). Detectable in amounts of 8 μ g. Gives a purple colour. Treatment with H_2O_2 produces a compound which travels more slowly in phenol and decomposes during the collidine run. It has not yet been found in nature by these methods.

Cysteic acid (1). Detectable in amounts of 5 μ g. Gives a blue colour. It is formed, apparently quantitatively, from cystine, by treatment with H_2O_2 . Old solutions of cystine may show the presence of cysteic acid. Traces of the latter, occasionally found in urine, may possibly have arisen from cystine by oxidation during storage. Urine from a patient with cystinuria, when treated with H_2O_2 , contains easily

detectable amounts of cysteic acid. This has occurred with the fresh urine as well as with urine that has stood until cystine crystals have deposited. No further evidence has therefore been secured in favour of the theory that the cystine is originally secreted in the form of a complex which decomposes on standing (Brand, Harris & Biloon, 1930). Cysteic acid is found in the hydrolysate of casein treated with H_2O_2 according to the method of Toennies (1942), and also in the hydrolysate of aged wool (Consden *et al.* 1946b).

Cysteine and cystine. These compounds are not usually seen in the chromatograms, at least when present in amounts of a few μ g. If large quantities are used, traces of cysteic acid may be detected and also spots due presumably to partial oxidation products. Consden *et al.* (1944), however, report a position for cystine on their chromatograms. The only apparent difference in their working conditions is in the use of the solvents in the reverse order, i.e. 'collidine' first, then phenol.

Diiodotyrosine (17). Detectable in amounts of 40 μ g. Gives a dull greenish purple, slightly duller and browner than that given by tyrosine. It readily couples with diazotized sulphanilic acid in Na_2CO_3 solution (Pauly reagent) to give a brownish red azo dye, presumably with the elimination of iodine. Previous development with ninhydrin does not interfere with the diazo reaction.

* $\beta\beta$ -*Dimethylcysteine* (penicillamine). It decomposes on the chromatogram when applied in amounts of a few μ g. Some $\beta\beta\beta'$ -tetramethylcysteine may be formed, however, and is then picked up in its accustomed position (*q.v.*).

**Djenkolic acid* (14). Detectable in amounts of 5 μ g. Gives a purple colour. Treatment with H_2O_2 produces a compound which travels more slowly in phenol and which decomposes during the collidine run.

Ethanolamine (48). Detectable in amounts of 5 μ g. Gives a purple colour. A serious loss of the material by volatilization always occurs if the paper is dried at 100°, and apparently to some extent when all the operations are carried out at room temperature. When the phenol run is carried out in the presence of 5*N*-HCl the substance finally occupies the position 48A. It is believed that ethanolamine is the substance in pathological urines, previously reported under the name 'fast-valine' (Dent, 1947b). It has also been found in the hydrolysate of gramicidin (Synge, 1945).

Ethanolamine-phosphoric acid (10)

Detectable in amounts of 10 μ g. Gives a purple colour; the colour produced at room temperature usually intensifies on heating. It is remarkably stable to acid hydrolysis, plenty of the material

surviving 6*N*-HCl for 24 hr. at 100°. It is unaffected by H₂O₂. A substance has been found in cancer tissue, intestinal mucosa and body fluids which matches the movement exactly on the chromatogram and also shows the above properties. Ethanolamine-phosphoric acid has been isolated from intestinal extracts by Colowick & Cori (1939).

Glucosamine (24). Detectable in amounts of 16 µg. Gives a purple colour. The ninhydrin reaction occurs as readily as with the common α-amino-acids. When the phenol run is carried out in the presence of the vapour from 1 : 1 (v/v) acetic acid it takes up the final position 24A. Glucosamine has been frequently found in the hydrolysates of naturally occurring proteins and is presumably derived from combined polysaccharide.

Glutamic acid (6). Detectable in amounts of 3 µg. Gives a purple colour. It often occurs as a streak along the direction of the dotted line, the bulk of the colour sometimes being collected at the two ends, almost as if it were trying to form two separate spots (compare the behaviour of aspartic acid). The lower part is always much redder in colour. At other times it may take up an even lower position on the map, thus causing serious danger of confusion with the other amino-acids in that region. This very low position is well illustrated in the photograph published by Martin (1947) of the chromatogram given by hydrolyzed gelatine. No reason can be suggested for these unpredictable anomalies of behaviour. Further difficulties suggestive of peptide formation arise when old solutions of glutamic acid are analyzed on the chromatograms. A two year old solution of glutamic acid in 0.4*N*-HCl has appreciable quantities of another ninhydrin-reacting substance which moves much more slowly than glutamic acid in phenol and is decomposed back into the latter on acid hydrolysis. Equally old solutions in 75 % (v/v) ethanol have given up to four spots widely separated on the paper. The main one of these, which travels to a position between leucine and ethanolamine, is detectable when the solution is only a few days old, and appears to be the γ-ethyl ester. Together with other anomalies in the behaviour of glutamic acid already reported in the literature these findings suggest that some fundamental facts in the simple chemistry of the compound still await discovery. Traces of glutamic acid are usually found in blood, urine, and plant tissues. Large amounts may be further liberated by mild acid hydrolysis even when only mere traces of glutamine are present. Presumably the glutamic acid in such cases is present in the form of a simple *N*-derivative, such as an acyl one (compare aspartic acid).

Glutamine (21). Detectable in amounts of 5 µg. Gives a purple colour. Incorrectly stated to move to the same position as alanine (Dent, 1947*a*), this substance moves to a position indistinguishable

from that believed also to be occupied by a peptide (see 'Under-alanine') containing serine and glycine. Owing to this behaviour the identification of glutamine must always be confirmed by hydrolysis, which should result in the disappearance of the glutamine with simultaneous appearance of at least a corresponding amount of glutamic acid but no increase in the serine or glycine. With this method it now appears that the substance found in this position in normal blood and urine is glutamine and not the peptide 'under-alanine' as previously claimed by the author (Dent, 1947*a*). Glutamine has also been detected in large amounts in pathological urines, in plant extracts and in Amigen.

**Glutathione* (4). Detectable in amounts of 10 µg. Gives a purple colour. A spot in this position has been given by tissue extracts. The position given by pure glutathione has not, however, been very exactly reproducible. Further work is required to determine the optimum working conditions for its identification.

Glycine (12). Detectable in amounts of 1 µg. Gives a slightly reddish purple colour. It is usually the main amino-acid in urine, but is usually found in any biological fluid. After its hydrolysis by acid, urine often shows a large increase in glycine, presumably obtained from hippuric acid.

Histamine (59). Detectable in amounts of 16 µg. Gives a dull greenish purple colour. Its identity can be confirmed by the bright red colour reaction which it gives with the Pauly reagent. The vapour from 1 : 1 (v/v) acetic acid does not affect its movement in phenol. If 5*N*-HCl be present, however, it is greatly slowed and ends up in the position 59A. It has been found in tissue extracts and possibly in pathological urines. There is a serious danger of losing the histamine owing to its volatility.

Histidine (27). Detectable in amounts of 20 µg. Gives a dull greenish purple colour. The strength of colour seems to be definitely less than that given by an equimolecular amount of the common α-amino-acids. Hence the presence of histidine is better detected by means of the Pauly reagent with which it gives an intense red colour. For this test the paper is freed from all traces of phenol or 'collidine' by leaving it overnight in the oven at 100° or by thorough washing with benzene or acetone. Previous use of ninhydrin does not interfere with the Pauly reaction. The position of histidine on the chromatogram varies appreciably with the nature of the 'collidine' used. Pure 2:4:6-collidine brings it to a position well below the level of methionine sulphoxide (no. 39 on the map), and also results in a much browner ninhydrin colour. Some impure 'collidines' of unknown isomer composition may raise it almost to the level of α-amino-*n*-butyric acid (no. 26). The movement in phenol is not slowed by the vapour of 1 : 1 (v/v) acetic acid. It is, however,

slowed to position 27A in the presence of 5N-HCl. Commonly found in biological fluids.

**Homocysteic acid* (3). Detectable in amounts of 10 μg . Gives a blue colour. It is readily formed from homocystine by the action of H_2O_2 . It may be confused with cysteic acid unless careful matching is done. Not yet found in nature.

**Homocystine*. This substance does not appear on the chromatogram owing to its decomposition.

Hydroxylysine (31). Detectable in amounts of 15 μg . Gives a dull purple colour. Its position on the chromatogram agrees with the rule that the addition of a hydroxyl group on to the β -carbon atom of an amino-acid (lysine in this case) lowers the R_F in phenol with little effect on the R_F in collidine. The amino-acid moves to position 31A when the phenol run is carried out in the presence of the vapour from 1:1 (v/v) acetic acid. It has not yet been found chromatographically in natural sources, although the photograph of the chromatogram from hydrolyzed gelatine published by Martin (1947) shows a suspicious smudge in the appropriate position.

Hydroxyproline (28). Detectable in amounts of 15 μg . Gives a brownish yellow colour. The spot usually touches that of alanine, but the two are easily distinguished by colour. Found a few times in urine, more often in hydrolyzed urines.

**Lanthionine* (8). Detectable in amounts of 6 μg . Gives a purple colour. Treatment with H_2O_2 produces a compound which travels more slowly in phenol and which decomposes during the collidine run. Not yet found in nature.

Leucine (45). Detectable in amounts of 10 μg . Gives a purple colour. Coincides with isoleucine and norleucine in the phenol-'collidine' chromatogram. Identification of a spot in this position is therefore best done after isolating the pure amino-acid by the cutting-out technique (Dent, 1947a; Consden *et al.* 1947). The material must then be run on a strip of paper (one-dimensional chromatogram) with butanol as solvent. The isomers are readily separated from each other in this way and should be identified by simultaneous comparison with standards of the pure amino-acids. Norleucine travels the fastest in butanol and is followed by leucine and then by isoleucine. Alternatively the original chromatogram could be run again with butanol as first solvent in the place of phenol. The positions with this solvent combination have not yet, however, been worked out accurately enough. In the absence of unidentified amino-acids whose behaviour in the solvents is unknown, the three leucines can be distinguished by a one-dimensional chromatogram run with benzyl alcohol or butanol on the original mixture (Consden, Gordon, Martin, Rosenheim & Synge, 1945). Leucine is commonly found in biological fluids.

Isoleucine (46). Detectable in amounts of 10 μg . Gives a purple colour. See 'Leucine' for identification. It is commonly found in biological fluids.

Lysine (58). Detectable in amounts of 15 μg . Gives a dull purple colour. Its spot always tends to be larger than the others and often shows streaking to the right. It takes up position 58A when the phenol run is carried out in the presence of the vapour from 1:1 (v/v) acetic acid. In the presence of 5N-HCl much greater slowing occurs in the phenol. It usually overlaps ornithine if present. For their identification in the presence of each other see 'Ornithine'. Commonly found in biological fluids.

Methionine (34). Detectable in amounts of 10 μg . Gives a purple colour. Overlaps with leucine, although it may be distinguished when present in high concentration. Its presence is best confirmed by the H_2O_2 technique (*q.v.*). Methionine slowly oxidizes to the sulphoxide if left to stand some days on the filter paper. It oxidizes more readily during the development of the chromatogram so that a pure sample of methionine usually gives two spots of about equal strength, one for the methionine and the other for its sulphoxide. This artifact of oxidation occurs mainly during the phenol run. The presence of methionine in the original sample may therefore be suspected whenever the sulphoxide is found in its characteristic position (no. 39) (see below for further details). The platinum reagent described by Consden *et al.* (1946a) can also be used to detect methionine. The test, however, is less sensitive than the ninhydrin colour reaction and cannot be applied after ninhydrin. It is not positive for the sulphoxide or sulphone, or for cystine. Methionine is only very rarely detectable in biological fluids.

Methionine sulphone (25). Detectable in amounts of 10 μg . Gives a purple colour. Is formed, apparently quantitatively, by treating methionine with an excess of H_2O_2 . Not believed to occur in biological fluids. It has been found in the hydrolysate of casein treated with H_2O_2 by the method of Toennies (1942).

Methionine sulphoxide (39). Detectable in amounts of 5 μg . Gives a purple colour. Treatment with H_2O_2 has hardly any effect on it, at most only a trace being further oxidized to the sulphone. In the presence of ammonium molybdate, however, H_2O_2 converts it quantitatively to sulphone. This serves as a most specific confirmatory test of its identity. It is only rarely found in biological fluids. When methionine sulphoxide is found in biological fluids, the question whether it was present as such in the original fluid or was formed from methionine during the phenol run can be settled by treatment with H_2O_2 without a catalyst. The sulphoxide, if present, survives the oxidation and is again found on the chromatogram. If only methionine is present it will all be oxidized to sulphone, and no sulphoxide is found. This

method has been used to confirm the excretion of sulphoxide, as such, in the urine, after giving methionine by mouth (Dent, 1947a).

**α-Methyl-α-amino-n-butyric acid* (49). Detectable in amounts of 10 μg. Gives a purple colour. As with *α-aminoisobutyric acid* which is also fully substituted at the *α*-carbon, the colour does not develop on standing a few days at room temperature. It slowly appears on heating to 100°. This behaviour enables it to be easily distinguished from neighbouring substances on the chromatogram. It is believed not to occur in nature.

Methylhistidine (51) (obtained from anserine). Detectable in amounts of 20 μg. Gives a dull greenish purple colour closely matching that given by histidine. It does not give any reaction with the Pauly reagent. Its spot partly overlaps that of proline from which it is easily distinguished by its colour. As shown on the map its relation to proline (no. 52) in a vertical direction is subject to a little variation. The position slightly above proline is more usual. A substance assuming exactly its position and matching its colour with ninhydrin has been found in several pathological urines and has appeared as the strongest spot in the chromatogram from a normal dog's urine.

**Monoiodotyrosine* (23). Detectable in amounts of 30 μg. Gives a dull greenish purple colour, closely matching that given by tyrosine. It readily reacts with the Pauly reagent to give a brownish red compound indistinguishable from that given by tyrosine. Its position is very close to, if not identical with, one of the radioactive spots found by Fink, Dent & Fink (1947) in the chromatogram of the hydrolysate of a rat's thyroid gland removed 24–48 hr. after an injection of ¹³¹I; otherwise no evidence has been obtained of its possible occurrence in nature.

**Norleucine* (47). Detectable in amounts of 10 μg. Gives a purple colour. See 'Leucine' for method of identification. Not believed to occur in biological fluids.

**Norvaline* (35) (*α*-amino-*n*-valeric acid, *α*-aminopentanoic acid). Detectable in amounts of 5 μg. Gives a purple colour. Not clearly distinguishable from valine, although a spot made up of both valine and norvaline may appear to be double. To distinguish between the two the substance giving the spot must be isolated by the cutting out technique (Dent, 1947a; Consden *et al.* 1947) and the substance run on a paper strip (one-dimensional chromatogram) with butanol. This readily separates them, the straight chain compound as usual travelling faster than its branched isomer. It is not believed to occur in nature.

Ornithine (41). Detectable in amounts of 10 μg. Gives a purple colour. The spot always overlaps that given by lysine if both substances are present together. The two substances appear at first

sight to be giving one large spot. The slightly different shades of the colour reaction can, however, usually be observed. For final confirmation of identity the chromatogram must be repeated with the vapour of 1 : 1 (v/v) acetic acid present during the phenol run. Under these circumstances the much greater slowing of the ornithine (to position 41A) compared with the lysine causes two distinct spots to be produced which are then easily identified. Ornithine has not yet been found in blood and urine.

Penicillamine. See ββ'-dimethylcysteine.

Phenylalanine (44). Detectable in amounts of 10 μg. Gives a greenish purple colour; with other 'collidines' the colour may be blue or even brownish. Addition of a trace of diethylamine to the cabinet is necessary for the phenylalanine to move to the position shown (Consden *et al.* 1947). In the absence of diethylamine or if only ammonia is added it invariably moves slower in the collidine and then overlaps the leucines seriously. It is often found in pathological urines.

Proline (52). Detectable in amounts of 8 μg. Gives a lemon yellow colour. It is often found in pathological urines.

Serine (9). Detectable in amounts of 2 μg. Gives a purple colour which may appear brownish when impure 'collidine' is used. It is often found in traces in normal urine, much larger amounts may be excreted in disease (Dent, 1947b).

**Serine-phosphoric acid* (2).

This was examined in the form of the Ba salt and was detectable in amounts of 10 μg. Gives a purple colour. It has given either a single or a double spot under apparently similar conditions. There is some evidence, however, that the part of the spot moving faster in phenol occurs when the concentration of ammonia is slightly greater. It has not yet been found free in nature. It is present in a combined state in casein and vitellinic acid.

Taurine (11). Detectable in amounts of 3 μg. Gives a purple colour. The ninhydrin reaction occurs as readily as with the common *α*-amino-acids. Owing to its characteristic position this is a most useful substance to add as a 'marker' to help in the identification of neighbouring amino-acids. Its position is not altered by the presence of acetic or hydrochloric acids during the phenol run. It is commonly found in normal blood and urine. In pathological urines it may be excreted in very large amounts to become the strongest spot on the chromatogram. This substance was alluded to under the name 'over-glycine' (Dent, 1947a) before it was identified. Paper chromatography probably pro-

vides the only simple specific test for this widely distributed substance. Many of the methods for determination of amino-acids in the urine will include taurine as contributing to the 'total amino-nitrogen'. Nitrous acid and formol methods will do so, and will therefore give quite incorrect results in some pathological urines containing an excess of taurine. The copper method (Albanese, Frankston & Irby, 1944) and the ninhydrin- CO_2 method (Van Slyke, MacFadyen & Hamilton, 1943) do not determine taurine.

* $\beta\beta\beta'\beta'$ -Tetramethylcystine (19) (oxidized penicillamine). Detectable in amounts of 10 μg . Gives a purple colour. Treatment with H_2O_2 and ammonium molybdate causes some of it to be oxidized to an unidentified substance which gives a typical ninhydrin reaction and moves to position 19A. It has not yet been found in biological fluids.

Threonine (16). Detectable in amounts of 10 μg . Gives a purple colour. The colour strength given by this amino-acid appears to be weaker than that given by the commoner α -amino-acids. The vertical position varies slightly with the nature of the 'collidine' used. With some crude batches it has been moved down nearly to the level of alanine. It is commonly found in biological fluids.

Thyroxine (42). Detectable in amounts of 60 μg . Gives an olive brown colour going greener on heating. Thyroxine moves so fast in the collidine that it may run into a brown impurity which usually shows on the finished paper in this region. This impurity is always formed during the phenol run and is subsequently carried forward in the collidine a little way behind its leading edge. When it does this, the thyroxine may be completely lost. There is clearly room for the development of a specific test for this substance which is more sensitive than the ninhydrin reaction, and which gives a colour which will show up against the occasional brown background. So far no success has been obtained in applying the nitrous acid reaction of Kendall & Osterberg (1919).

Tryptophan (32). Detectable in amounts of 20 μg . Gives a purple colour. It is not usually present in blood and urine, although it has been found in the urine of patients with severe liver damage (Dent, 1947b). Not found in acid hydrolysates of proteins owing to its destruction by the acid.

Tyrosine (18). Detectable in amounts of 15 μg . Gives a dull greenish purple colour. Because of the very characteristic and unambiguous position of its spot, tyrosine is a most useful 'marker'. It gives, in similar strength, a brown colour with the Pauly reagent. A sample of 'pure L-tyrosine' from a reputable manufacturer was found to contain a large quantity of leucine. It is commonly found in biological fluids.

'Under-alanine' (22). This name was given by Dent (1947a) to a substance present in the urine of

a patient with Fanconi syndrome and cirrhosis of the liver. The substance gave a reddish purple colour with ninhydrin and, on hydrolysis of the urine, the subsequent chromatogram contained no 'under-alanine' and showed a remarkable increase in the content of serine. Experiments on the isolated substance suggested that it might be the tripeptide serylglycylglycine. This substance has not been encountered again, although there is no reason to doubt the original finding. It is now known that both glutamine and a substance in normal blood and urine and in some other pathological urines also move to a very similar position to that of the tripeptide. It is now considered that the substance in normal blood and urine is glutamine and not the tripeptide, as claimed by the writer (Dent, 1947a). It is considered safe to say that a spot in this position is due to glutamine if it disappears on hydrolysis to liberate at least an equivalent amount of glutamic acid without increasing the serine content perceptibly.

Valine (36). Detectable in amounts of 3 μg . Gives a purple colour. Nearly always found in normal and pathological blood and urine and in tissue extracts of plants and animals.

Commonly occurring unidentified substances

'Fast-aminobutyric acid' (50). This is an unidentified substance which has been frequently found in plant extracts and in blood and urine. It is the substance numbered 24 in the photograph of the chromatogram of the potato extract published by Dent *et al.* (1947). It is stable to acid hydrolysis and to H_2O_2 and is not slowed during the phenol run by working at an acid pH. It is therefore believed to be a monoaminomonocarboxylic acid. It does not react with diazotized sulphanilic acid or with *p*-dimethylaminobenzaldehyde.

'Fast-arginine' (60). This is an unknown substance giving a typical purple colour with ninhydrin. It is stable to acid hydrolysis, and appeared on one occasion to concentrate in a fraction precipitated with phosphotungstic acid. When the phenol run is carried out in the presence of the vapour from 1 : 1 (v/v) acetic acid it is slowed to the position 60A. It is therefore believed to be a new basic amino-acid. It has been found several times in blood and tissue extracts and was one of the main amino-acids to be passed in the urine of a patient with Fanconi syndrome. Its movement on the chromatogram suggests that it may be the guanidine compound derived from lysine, i.e.

'Green-spot' (53). An unidentified substance giving a bright green colour with ninhydrin. It has been seen in many normal and pathological urines. It survives hydrolysis with 6N-HCl for 24 hr. at 100°.

'Nephrosis-peptide' (61). An unidentified substance found in large amounts in the deproteinized urine in one out of three patients with nephrosis. It was found in the blood of one of the patients who was not passing it in the urine. It gives a typical purple colour with ninhydrin, and on hydrolyzing liberates most of the common amino-acids. Traces of a substance moving to a similar position have occasionally been seen in other urines.

Amino-acids not shown on the map

Thiolhistidine gives bands in phenol of R_F 0.22. In collidine, however, much decomposition appeared to take place, for the substance which gave a very weak ninhydrin colour at R_F 0.42 no longer gave the Pauly reaction.

Dihydroxyphenylalanine gave bands in phenol and collidine (R_F 0.33 and 0.44 respectively) but has never been seen in a two-dimensional chromatogram. Presumably it decomposes during the longer time necessary for this process.

Peptides not shown on the map

Anserine moves readily on the chromatogram to a position near proline. The ninhydrin reaction, however, is so weak that it is unlikely to be picked up unless present in very large amount. Anserine (75 μ g.) has been run on a two-dimensional chromatogram without being subsequently found. It is more easily seen on one-dimensionals. Under such circumstances it gives a dull greenish colour with ninhydrin.

Purely synthetic peptides do not come within the scope of this paper.

Substances that do not react with ninhydrin

Pyrrolidone carboxylic acid does not give a ninhydrin reaction and its movement on the chromatogram has not yet been traced.

This is no place to mention in detail substances which do not react with ninhydrin. It is, however, repeated that the previous suggestion of the writer (Dent, 1947a) that the paper chromatographic method might be of very general application to organic and inorganic substances has been fully confirmed in subsequent trials. The easy separation of chlorides, bromides and iodides by collidine is quoted as a further example of the latter. As a result of this it is useful to bear in mind that the same paper chromatogram which has been run on, say, a specimen of urine, can often, after the amino-acids have been identified by development with ninhydrin, be again used for the identification of other substances present, e.g. urea, which moves to a position below tyrosine on the map. There is very great scope therefore for the extension of the

method to simultaneous qualitative analysis covering a wide range of compounds likely to be present in biological fluids.

DISCUSSION

The chief point relative to the more general use of the paper chromatographic method concerns the question of specificity. How far is one justified in assuming that a spot in the exact position of, say, alanine, is in fact due to alanine?

First must be considered the question of the specificity of the ninhydrin colour reaction. There is still much confusion on this subject. For instance, Schmidt (1944) still states erroneously that β - and γ -amino-acids do not give the reaction. As shown here, where only reactions leading to good colour strength are referred to, many primary amines, as well as the β -, γ -, δ -, and ϵ -amino-acids will give typical reactions, the rate of development of the colour, the strength and the actual shade being identical with that given by α -amino-acids. However, with mixtures obtained from natural sources only a limited number of such substances is likely to be present. The simple amines are readily detected by subsequent tests depending on the great difference in rate of movement in phenol caused by a difference in acidity (e.g. glucosamine). Substances like taurine may be confused with amino-acids, although it seems likely that sulphonic acids will always be detectable by their unusually fast movement in the 'collidine'. The δ - and ϵ -amino-acids have so far only been reported to be formed as the result of putrefaction (Neuberg, 1911 and Ackermann, 1910, respectively).

Secondly, the question of the accuracy of the position matching arises. Here it must be repeated that there is no need to determine R_F values when enough different amino-acids are present to enable identification from the pattern of the chromatogram. With very few exceptions the latter is quite extraordinarily constant, since day to day variables always and variations in the isomer composition of the collidine nearly always affect the R_F values in the same proportions and do not therefore alter the pattern. When a spot giving the typical ninhydrin reaction occurs in the pattern in the exact position of alanine, then the probability is overwhelming that the unknown substance is in fact alanine. A final check must still be done, however, by adding pure alanine to the mixture and then observing that under these identical conditions the pure substance runs exactly with the unknown, and that it is quite stable to hydrolysis. Hydrolysis is most essential, as there are so many possible peptides that every position occupied by an amino-acid may presumably be overlapped by unknown peptides. These may give typical ninhydrin reactions but can be decomposed on suitable hydrolysis. For this reason no

certain identification is possible by simple matching of position of substances which are unstable to hydrolysis. The writer's confusion of glutamine with a peptide containing serine and glycine serves as a personal reminder of this fact.

When the unknown spot in the hydrolyzed solution has been matched with that of a known amino-acid, the next point to be considered concerns the fact or likelihood of another simple substance moving to the same place. This is a far more serious possibility with amino-acids containing more than four carbon atoms (i.e. valine and upwards), since there is a greatly increased chance of isomeric or of totally unrelated substances coinciding. Hence with such substances the identity should be confirmed by isolation by the cutting-out technique (Dent, 1947a; Consden *et al.* 1947) followed by matching in other solvents. So far, butanol is the only solvent that need be regularly used for this purpose, but the use of any number could be envisaged in case of ambiguity, since it is little trouble to isolate enough for 10–20 trials.

Finally, other tests, more specific than the ninhydrin reaction, can be applied directly to the spot on the paper, as described for histidine, tyrosine and citrulline. Stability to H_2O_2 would also exclude the presence of dicovalent sulphur. Basic amino-acids are greatly slowed in phenol by acetic or hydrochloric acid vapours. There is much scope for the development of further tests for individual substances.

Bearing all this in mind it must still be admitted that there remains a possibility of confusion between a well-known substance and another whose presence has not been suspected and which may happen to coincide on the chromatogram. The risk is believed small and will diminish as our knowledge of natural substances increases.

It will be noted that two-dimensional chromatograms are exclusively recommended when a mixture of quite unknown composition is to be analyzed. One-dimensionals are only safe when the mixture contains a limited number of amino-acids all of known composition and whose behaviour on the paper has already been investigated, as in the analysis of many protein hydrolysates. For several reasons, one-dimensional chromatography is not safe in the investigation of the non-protein nitrogen fractions of tissues or in the analysis of blood and urine, in all of which new amino-acids have been found with embarrassing frequency. First, there is the phenomenon of 'double overlapping', illustrated in Fig. 3. Many instances of this have been encountered in practice. Secondly, there is the impossibility when the mixture contains very many amino-acids of obtaining good separation of the amino-acids from each other on one-dimensionals. This may be readily surmised from the published photograph of the two-dimensional paper chromatogram obtained by Dent

et al. (1947) from an extract of potato tuber. This extract contained at least 24 ninhydrin-reacting substances, probably all amino-acids. Finally it is often desired to run very large amounts of the mixture on the chromatogram in order to show up constituents present only in relatively small quantities. This can be done on two-dimensionals well beyond the point where in one-dimensionals the same quantity would show as a continuous streak of colour which would be quite impossible to interpret.

Fig. 3. Diagram of paper chromatograms drawn to illustrate the phenomenon of 'double overlapping'. The four circles (A, B, C and D) represent the positions occupied by four hypothetical amino-acids after running as usual on the paper square. One-dimensional chromatograms in phenol and collidine, run with the solvents in the same directions as on the square, are shown below and to the side respectively. In such a case the square would readily resolve a mixture containing some or all of the substances A, B, C or D. The two one-dimensionals if run instead of the square would show no qualitative differences between mixtures containing A and D; B and C; A, B and D; A, C and D; B, A and C; B, D and C; A, B, C and D, since all would appear to contain only two substances. Similar difficulties occur with mixtures only likely to contain three substances, provided that they move to positions similar to three of those in the figure, and in a mixture containing twenty or more amino-acids many instances of double overlapping may occur at the same time on different parts of the chromatogram.

The comparison of an unknown substance with a pure compound which is believed to be identical can be made extraordinarily accurate when the latter reference compound can be obtained in radioactive form. The latter is added to the mixture in trace amounts before they are run together on the chromatogram. The final position of the unknown can then be developed as usual with ninhydrin, and the position of the added pure substance determined by means of a radio-autograph, i.e. by holding the paper against a photographic plate for a sufficient period of

time. The two compounds are thus developed independently of one another. If they are one and the same they will coincide exactly, not only in their average positions but also in the size and shape of the spot. The slight irregularities which always occur in the shape must be exactly reproducible. The final identification has the finality of a 'finger-print' method. This method has been used already by Fink *et al.* (1947) in the case of diiodotyrosine containing ^{131}I .

In practice, with substances of natural origin and the solvent combination described here, the only real confusion in the identification of simple amino-acids in the absence of peptides occurs in the cases of leucine and isoleucine and of citrulline and β -alanine. The methods for distinguishing these are given in the experimental section.

It is conceded that actual isolation in quantity followed by the classical methods of chemical identification should also be undertaken when possible, and is advisable as an independent method of confirmation when claims of the discovery of new amino-acids are made. However, it is held strongly that paper chromatography has a place of its own in amino-acid problems, at least as important as that of the older methods. The latter are themselves often subject to serious criticism when applied to substances separated from mixtures containing many closely related compounds. Certainly as far as concerns the investigation of mixtures, the chromatographic method is far more specific than any chemical or microbiological method in use at the present time.

Finally, it is urged that a new name be found for a technique which is bound to have wide application as a general method of chemistry and which can only be adequately described now as 'partition chromatography using filter paper'. The name 'papyrography' is submitted for consideration.

SUMMARY

1. The two-dimensional filter-paper method of partition chromatography has been applied to some sixty amino-acids and other ninhydrin-reacting substances, using the pair of solvents, phenol and 'collidine'.

2. A map is shown giving the positions taken up by these substances on the paper square.

3. With additional tests the method can be applied to the identification of all the ninhydrin-reacting substances likely to be present in biological fluids.

4. The ninhydrin colour reaction as used here is positive for many aliphatic amines and also for β -, γ -, δ -, and ϵ -amino-acids.

5. Peculiarities in the behaviour of glutamic acid, and to a lesser extent of aspartic acid, suggest that there are unknown facts in the chemistry of these substances.

6. Deaminized casein when hydrolyzed contains only a trace of lysine and an ample quantity of α -amino- ϵ -hydroxycaproic acid.

7. Apart from the common amino-acids the following substances have been found in biological fluids by this method of analysis: β -alanine, α -amino-*n*-butyric acid, γ -aminobutyric acid, ethanolamine, ethanolamine-phosphoric acid, histamine, methionine sulphoxide, methylhistidine. In addition, mention is made of four common but unidentified substances that have been encountered. One ('fast-aminobutyric acid') appears to be a monoaminomonocarboxylic acid, another ('fast-arginine') a new basic amino-acid.

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