The combination of these procedures was extensively used in biochemical studies, dealing with the enzymic oxidation of certain hydroxy acids[19]. References to preparative methods of various keto acids are also listed in this paper.

SUMMARY

A systematic analysis of keto acids and other carbonyl compounds in biological material was based on the following procedures: (1) their conversion to DNPH derivatives under mild conditions, (2) preliminary chromatographic separation into acidic and non-acidic DNPH derivatives, (3) isolation of each component by paper chromatography and (4) identification by means of rechromatography and catalytic reduction to the corresponding amino derivative. The method is quantitative for keto acids and its accuracy is between 10–20%.

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THE QUANTITATIVE DETERMINATION OF AMINO ACIDS BY PAPER CHROMATOGRAPHY*

A SOLVENT TO REPLACE PHENOL

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Water-saturated phenol, used in an ammoniacal atmosphere, has been found to be a satisfactory first solvent for two-dimensional qualitative separations of amino acids on paper chromatograms, for which *n*-butanol/glacial acetic acid/water (the organic layer of a mixture of 4:1:5 parts by volume) is used as the second solvent. To obtain

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good quantitative recoveries of amino acids separated by this solvent system it is necessary to remove the phenol from the chromatograms before drying them, by washing with ether. Otherwise, losses of 10–30% of each amino acid occur during the drying process due to the interaction between the amino acids and phenol at 50–60°C, which is the optimal drying temperature for phenol (FOWDEN[1]). Under carefully controlled conditions, the amount of each amino acid destroyed is constant to within 3% so that, if chromatograms of standard amino acid solutions are run and dried together with the experimental chromatograms, it is possible to apply factors to correct for any losses. Nevertheless these losses were considered to be too great for the technique to be entirely satisfactory as a quantitative procedure. A method was required for routine amino acid analyses involving numerous sheets of paper 45–60 cm square, and losses could not be avoided by removing the phenol with ether before drying because of the fire hazard that that would incur. A study of solvents which might be used instead of phenol was therefore undertaken.

EXPERIMENTAL

Several of the solvents commonly used in chromatography were tried in varying combinations and, of these, the most satisfactory was found to be a one-phase solvent consisting of 5 parts by volume of *n*-butanol, 3 parts by volume of methyl ethyl ketone and 1 part by volume each of water and 17 *N* ammonia. (This solvent will be referred to henceforth as butanol-ketone.) Butanol-ketone has the general advantages over phenol in that it is less unpleasant to handle, it can be removed from the chromatograms in three hours at 40°C, and losses of amino acids during the running and drying processes are small (0–4%). Good qualitative separations were obtained on 35 cm squares of Whatman 3MM paper, by using this solvent for the first dimension and *n*-butanol/acetic acid/water (the organic layer of the 4:1:5 parts by volume mixture — to be referred to as butanol-acetic acid) for the second dimension. In order to maintain a strongly ammoniacal atmosphere in the tanks with the first solvent, beakers containing some of the solvent were placed at the bottom of the tanks. The amino acid solution (50 μl), containing 1–2.5 μg of α-amino-nitrogen of each amino acid, was placed 10 cm in from two adjacent edges of the paper to give a spot of diameter 4–5 mm. Technical grade solvent reagents were used and were not redistilled or otherwise purified. At 20–21° C, using descending solvent systems, 34 hours are required for full resolution in the first dimension (*i.e.* 4–4.5 times the period taken by the solvent to reach the bottom of the paper) and 20 hours are required for the second dimension (1.3 times the period for the solvent to reach the bottom of the paper). The solvents are removed by drying the papers in an air current at 40° C for 3 hours and 5 hours, respectively.

Separations satisfactory for quantitative work were obtained on 50 cm square sheets of Whatman 3 MM paper. The amino acids were dissolved in 10 volume hydrogen peroxide and kept at 30°C for 30 minutes before being applied to the chromatograms. This preliminary oxidation is necessary to convert cystine and cysteine, which streak badly in butanol-acetic acid, to cysteic acid. The oxidation also converts methionine to its sulphoxide and sulphone which have very similar R_F values in butanol-acetic acid, but separate in both butanol-ketone and phenol. The amino acid solution (50–100

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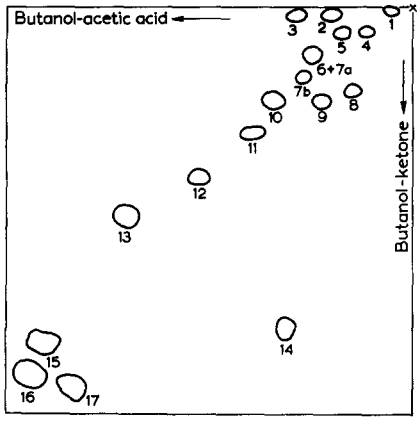

Fig. 1

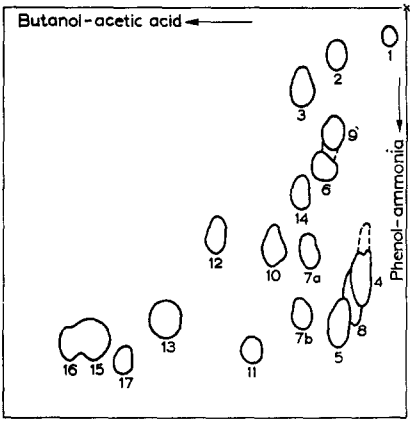

Fig. 2

Figs. 1 and 2. Amino acids separated by the solvent pairs butanol-ketone and butanol-acetic acid (Fig. 1) and water-saturated phenol/ammonia atmosphere and butanol-acetic acid (Fig. 2). 1, Cysteic acid; 2, Aspartic acid; 3, Glutamic acid; 4, Lysine, 5, Arginine; 6, Glycine; 7a, Methionine sulphone; 7b, Methionine sulphoxide; 8, Histidine; 9, Serine; 10, Alanine; 11, Proline; 12, Tyrosine; 13, Valine; 14, Threonine; 15, Isoleucine; 16, Leucine; 17, Phenylalanine.

μl) containing 2–5 μg of α-amino-nitrogen of each amino acid is applied to the paper in a spot of diameter 7–8 mm, and at least 10 cm in from two adjacent edges of the paper. An accurately scaled tracing of such a chromatogram is given in Fig. 1. The spots are sharply defined and show no evidence of the bearding or tailing which are apt to occur in phenolic solvents. They may be compared with those in Fig. 2, which represents a similarly scaled chromatogram of the same amino acid mixture run first in water-saturated phenol in an ammoniacal atmosphere and then in butanol-acetic acid. The butanol-ketone, butanol-acetic acid solvent pair have the advantage that they separate leucine, isoleucine, lysine, arginine and histidine, so that it is unnecessary to run additional one-dimensional chromatograms to separate them, as is the case when phenol and butanol-acetic acid are used. However, the individual concentrations of glycine and methionine cannot be determined directly with the first solvent pair, since methionine sulphone runs with glycine in butanol-ketone. One-dimensional chromatograms of an unoxidized solution of the amino acids must be run in butanol-acetic acid. In this solvent methionine runs together with valine so that a value for methionine plus valine can be obtained. Using this, and the values determined from the two-dimensional chromatograms for glycine plus methionine and for valine alone, the individual amounts of glycine and methionine present may be calculated. Glutamine and asparagine can be detected on butanol-ketone, butanol-acetic acid chromatograms but as glutamine partly overlaps glycine and arginine, and asparagine partly overlaps lysine and arginine, the separation is inadequate for quantitative work. This difficulty may be overcome by converting the amides to their corresponding acids by heating the amino acid solution containing them for 3 hours at 100°C with 1 *N* hydrochloric acid, before applying it to the chromatograms. The amide nitrogen may then be determined separately by estimating the ammonia produced during the acid hydrolysis by a nesslerization procedure.

The order in which the amino acids separate in butanol-ketone is always the same

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but the relative distances travelled by individual acids may vary up to 4%. The R_F of phenylalanine, which moves the most rapidly of the amino acids studied, is 0.25. Because the R_F values are so low it has been found more useful to express the distances travelled by the amino acids from the origin as ratios of the distance between the origin and phenylalanine, *i.e.* as R_Φ values. R_Φ values derived from several one-dimensional chromatograms are given in Table I. R_Φ values derived from two-dimensional chromatograms for which butanol-acetic acid is used as the second solvent are substantially the same. However, if the solvents are used in the reverse order it is found that, while the R_Φ values for the other amino acids remain unchanged, that of threonine is decreased from 0.83 to 0.73. This change is of no importance for two-dimensional chromatograms since on these threonine is equally well separated from other amino acids irrespective of the order in which the solvents are used, but it is of significance for one-dimensional separations in butanol-ketone. When threonine has an R_Φ of 0.83 it cannot be separated from isoleucine ($R_\Phi = 0.87$), but when it has an R_Φ 0.73 a separation from isoleucine can be completely effected. It was thought that traces of acetic acid retained by the paper after the butanol-acetic acid had been dried off might have influenced the threonine. Papers were sprayed with a 10% solution of glacial acetic acid and then dried before being used for one-dimensional chromatograms; the R_Φ of threonine was 0.79 and a better separation of threonine from isoleucine was obtained. However, the lower two-dimensional R_Φ value for threonine (*i.e.* 0.73) can be obtained by first allowing butanol-acetic acid to run down the full length of clean sheets and then drying the sheets at 40°C before using them for separations in butanol-ketone. It would seem that factors other than traces of acetic acid are responsible for the lower R_Φ value.

TABLE I

R_Φ VALUES OF AMINO ACIDS IN THE SOLVENT *n*-BUTANOL: METHYL ETHYL KETONE : WATER : 17 *N* AMMONIA = 5:3:1:1 PARTS BY VOLUME

Amino acid	R_Φ	*Amino acid*	R_Φ	*Amino acid*	R_Φ
Cysteic acid	0.01	Asparagine	0.10	Serine	0.26
Glutamic acid	0.02	Methionine sulphone	0.13	Proline	0.32
Aspartic acid	0.02	Hydroxyproline	0.13	Tyrosine	0.44
Lysine	0.08	Glycine	0.14	Valine	0.60
Arginine	0.08	α-Aminobutyric acid	0.19	Methionine	0.62
Ornithine	0.09	Methionine sulphoxide	0.21	Threonine	0.83
Citrulline	0.09	Histidine	0.24	Isoleucine	0.87
Glutamine	0.10	Alanine	0.25	Leucine	0.95
				Phenylalanine	1.00

Using such sheets of Whatman 3MM paper pre-washed in butanol-acetic acid proline, tyrosine, valine, threonine and isoleucine can be separated on a preparative scale on one-dimensional chromatograms by butanol-ketone.

The amounts of the amino acids separated on chromatograms were determined by the method detailed by FOWDEN[2], except that the MOORE AND STEIN[3] colorimetric method was replaced by that of YEMM AND COCKING[4], and that proline was determined by the CHINARD[5] method. The Yemm and Cocking procedure was slightly modified. A more concentrated citric acid buffer solution (65 g/l) had to be used, partly to cor-

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rect for the alkali added to remove any ammonia present and partly to improve the development of the purple derivative which was found to be adversely affected by the paper. Because of the presence of paper it was also found necessary to extend the heating period to 30 minutes and to cool the tubes for 10 minutes in an iced water bath. Double volumes of all the reagents were used so that the pieces of paper were fully submerged. The optical density (O.D.) values given by the paper blank solutions were fairly low (a piece of paper 2.5 cm square giving a colour equivalent to 0.5 μg of α-amino nitrogen) and consistent. Experimental readings were corrected for the appropriate blank values. It is inadvisable to use the solvents in the reverse order for quantitative work (that is, using butanol-acetic acid first and following it with butanol-ketone), as the paper blank values, though still consistent, are 3–4 times higher. It would appear that the ammoniacal solvent reacts on the paper to form a ninhydrin-sensitive compound which is not removed even by prolonged drying at 40°C. This difficulty is completely avoided if butanol-ketone is used first and followed by butanol-acetic acid.

When determined in the presence of paper the maximum colours given by the ninhydrin derivatives were not, as they are in paper-free solution, the same for equal amounts of all amino acids (YEMM AND COCKING[4]). Known amounts of each amino acid were pipetted individually onto 2.5 cm squares of Whatman 3MM paper, the pieces of paper were dried, and the O.D. of the solutions of the ninhydrin-amino acid derivatives were determined by the Yemm and Cocking procedure. These O.D. values (O.D.(a)) were then compared with those obtained for equal amounts of the amino acids when determined in paper-free solution (O.D.(b) values). Column A, Table II, records the values for (O.D. (a))/(O.D. (b)) $\times$ 100, and shows the effect of the paper on the O.D. values. Since the concentrations of the amino acids separated on chromatograms were also determined in the presence of paper, the recovery values given in Column B, Table II, were obtained by comparing the O.D. values for the amino acids from chromatograms (O.D. (c)) with those for the same amounts of the amino

TABLE II

Amino acid	*A* % *Recovery from solvent-free paper**	*B* % *Recovery from chromatograms**
Alanine	100.0 ± 0.91	97.0 ± 2.8
Arginine	98.0 ± 1.23	98.0 ± 2.0
Aspartic acid	100.0 ± 1.31	97.0 ± 2.7
Cysteic acid	94.0 ± 1.14	96.5 ± 1.7
Glutamic acid	100.0 ± 1.51	95.5 ± 2.1
Glycine	97.0 ± 1.45	97.0 ± 1.7
Histidine	75.8 ± 1.35	95.0 ± 2.9
Isoleucine	99.0 ± 1.18	98.5 ± 1.9
Leucine	100.0 ± 0.81	97.0 ± 2.6
Lysine	100.0 ± 0.08	98.5 ± 1.2
Methionine	96.0 ± 0.94	95.0 ± 3.0
Phenylalanine	98.5 ± 1.24	99.0 ± 2.6
Proline	66.0 ± 0.48	99.0 ± 1.1
Serine	97.0 ± 1.13	96.5 ± 1.6
Threonine	98.0 ± 0.96	99.5 ± 1.6
Tyrosine	96.0 ± 0.82	96.0 ± 2.6
Valine	99.0 ± 0.72	100.0 ± 2.3

* See text for a full explanation of the derivation of these recovery values.

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acids added directly to paper, *i.e.* with O.D. (a) values. Figures in Column B are, therefore, (O.D. (c))/(O.D. (a)) × 100. They are the arithmetic mean of twelve replicate values ± the standard deviation.

DISCUSSION

Although the recovery values are not in all cases as high as those obtained by amino acid separation on Dowex-50 columns, they are probably sufficiently quantitative for most routine analyses of amino acids. For experiments in which recovery values of 90–98% are adequate, it is unnecessary to run chromatograms of standard solutions since the amounts of the amino acids on experimental chromatograms can be determined, at that level of accuracy, by comparison with standard O.D.(a) values. Should a greater accuracy be required, standard chromatograms can, of course, be run together with the experimental chromatograms so that corrections may be made for losses incurred on the chromatograms. This, however, doubles the work involved and it is suggested that for much routine analysis only experimental chromatograms need by run, so avoiding the additional expenditure of materials, time and labour.

While the butanol-ketone, butanol-acetic acid solvent system was primarily designed for the analysis of plant proteins, it is considered that it might equally well be used for many other amino acid mixtures. The procedure described in this paper has, to date, been successfully used for the analysis of ten maize, sunflower and cotton seed proteins. A paper embodying the results of this work is being prepared and will be published elsewhere.

SUMMARY

A one-phase solvent consisting of *n*-butanol, methyl ethyl ketone, 17*N* ammonia and water (5:3:1:1, v/v) has been used instead of phenol in two-dimensional paper chromatography for which *n*-butanol–acetic acid–water (4:1:5, v/v) is used as the second solvent. Clear separations, allowing quantitative recoveries, of the amino acid constituents of proteins have been obtained. Losses of amino acids during chromatography and subsequent estimations by a ninhydrin colorimetric procedure are small, being mainly from 1–4%.

The R_F values of 25 naturally occurring amino acids are given for this aqueous butanol–ketone –ammonia solvent.

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