The Separation and Determination of Microquantities of Lower Aliphatic Acids, including Fluoroacetic Acid*

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(*Received* 6 *May* 1955)

Although various methods of paper-chromatographic separation of fatty acids, using either their salts or the corresponding hydroxamic acids, have been described (Block, Durrum & Zweig, 1955), several problems still remain unsolved. Thus the quantitative separation of microquantities of formic and acetic acids by chromatography on paper has met so far with little success (Brown, 1950; Hall, 1950) and no method has yet been found for the separation and quantitative estimation of fluoroacetate, which in biological fluids is always accompanied by acetate. Fluoroacetate is a substance of great biochemical interest and is widely used as rodenticide. The analysis of foodstuffs, body fluids and tissues for this poison is therefore often desirable. Chromatographic separation of fluoride and fluoroacetate on a silicic acid column has been attempted by Ramsey & Clifford (1949), and the applicability to fluoroacetate of the lanthanum nitrate method for acetate has been reported by Hutchens & Kass (1949). However, these procedures do not permit the quantitative determination of microquantities of fluoroacetate, such as can be expected in biological material.

In the present investigation two problems have been studied: (*a*) the paper-chromatographic separation of formate and fluoroacetate from acetate and the other lower fatty acids; (*b*) the quantitative determination of microquantities of fatty acids, including fluoroacetate, from paper chromatograms.

After many unsuccessful trials with the sodium or ammonium salts of these acids our efforts were concentrated on the study of the corresponding hydroxamic acids. Appropriate conditions were found for the separation of formhydroxamic and fluoroacetohydroxamic acids from the hydroxamates of other lower fatty acids. In addition, a new colorimetric procedure for the quantitative determination of these derivatives has been found, with a lower limit of sensitivity of about 0·5 μg./ml. of final solution. The principles of the new method are described in the present paper. Application to body fluids and tissue extracts will be reported in a later communication.

* Presented at the Third International Congress of Biochemistry, Brussels, August 1955.

MATERIALS AND METHODS

Benzohydroxamic acid, m.p. 131°, was prepared according to Hauser & Renfrow (1943). α-Naphthylamine was recrystallized twice from light petroleum (b.p. 60–80°). All solvents used were commercial products, purified by distillation.

Preparation of hydroxamic acids. A portion of the solution to be examined, representing about 1 mg. of organic acid, was made alkaline with ammonia, the solution evaporated to dryness and the residue extracted twice with 1 ml. of 0·4N-HCl in ethanol. Ethereal diazomethane was added until a persistent yellow colour was obtained, and after 10 min. standing the excess of reagent was destroyed by addition of a few drops of 0·4N-HCl in ethanol. Hydroxylamine reagent (equal vol. of 2N hydroxylamine sulphate and 3·5N-NaOH) (2 ml.) was added and the mixture left at room temp. for 15 min. The solution was then concentrated on a water bath to a definite volume and a portion, representing 5–50 μg. of organic acid, spotted on Whatman no. 1 paper.

Paper-chromatographic separation of hydroxamic acids. The spots were first developed with a basic solvent mixture (solvent B): 95% ethanol, containing 5% NH_3–pyridine–water (3:1:1, by vol.). The paper was then dried, turned through 90° and developed with an acidic mixture (solvent A): 95% ethanol–dioxan–water–acetic acid (60:20:19:1, by vol.).

The R_F values given in the tables were determined in ascending chromatograms, which were developed for about 5 hr. (solvent front 12–15 cm. from the original spots).

Qualitative determination and location of spots. The procedure of Fink & Fink (1949) for the detection of hydroxamic acids was modified by the use of 1% $FeCl_3$ in 95% ethanol, containing 0·1% HCl. This reagent is preferable to the butanol solution, since drying takes place much faster and the staining is much clearer. The hydroxamic acids appeared as violet spots on a yellow background. This procedure was applied for the measurement of R_F values. Also, in analytical runs, a sample of the substance to be determined was run at the side of the paper, which was cut off and stained with $FeCl_3$ for location purposes. After some experience, however, this becomes unnecessary.

Colorimetric analysis of hydroxamates by conversion into nitrite. For quantitative analysis of the hydroxamic acids after chromatographic separation an area of 2 × 2 cm., containing the unstained material, was extracted with 5 ml. of 5% sodium acetate solution at room temp. for 15 min. Then 0·5 ml. of 1% sulphanilic acid in 25% (v/v) aqueous acetic acid was added, followed by 5 drops of I_2 solution (1·3 g. of I_2 in 100 ml. of acetic acid) and the mixture left for 4 min. Excess of I_2 was destroyed with

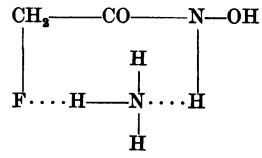
2–3 drops of 0·1 N-$Na_2S_2O_3$, then 2 drops of naphthylamine reagent (0·6% α-naphthylamine in 30% (v/v) acetic acid) and water to 6 ml. were added. The solution was decanted from the paper and read after 10 min. in a Klett–Summerson photoelectric colorimeter, using filter no. 52 (peak transmission 520 mμ.).

RESULTS

Separation of acetate and fluoroacetate

The sodium or ammonium salt of fluoroacetic acid is not suitable for chromatography, since none of the common indicators can be used for spot detection. With the corresponding hydroxamic acid no separation from acetohydroxamic acid was obtained with the usual acid solvent mixtures (Thompson, 1951). When the solvent contained pyridine, a higher R_F value was observed for fluoroacetohydroxamic acid than for the acetate derivative, but the former could not be separated from propionate and butyrate.

It was then thought that the greater acidity of the fluoro compound might permit separation from the weaker aliphatic hydroxamic acids in a suitable pH range, where only the fluoro compound undergoes dissociation. However, the results in Table 1 show that within the pH range 5–11 separation could not be effected. When, however, instead of the various possible factors was made and the following results were obtained: (*a*) ammonia in ethanol, without further components, produces separation of fluoroacetate and acetate. However, considerable trailing ensues and makes this simple solvent unsuitable for quantitative analysis. On addition of pyridine, the spots become concentrated. (*b*) When the ammonia molecule is gradually substituted by alkyl groups, the difference in R_F value between acetate and its fluorine derivative decreases more and more and disappears completely for trimethylamine (see Table 1). The difference between formohydroxamic and acetohydroxamic acids is of the same order in ammonia, mono- and di-methylamine, but is much less in trimethylamine. It can thus be concluded that at least one hydrogen atom must be present in the base. With fluoroacetohydroxamic acid it appears possible that intramolecular hydrogen bonding of the type (I) is responsible for the very low migrating



(I)

Table 1. *R_F values of normal aliphatic hydroxamic acids in various solvent mixtures*

The solvents were composed as follows: 1, 95% ethanol–pyridine–0·01 M phosphate buffer (60:20:20, by vol.), apparent pH of mixture 5·6; 2, as 1, apparent pH 8·0; 3, as 1, but with 0·01 M borate buffer, apparent pH 9·5; A, 95% ethanol–dioxan–water–acetic acid (60:20:19:1, by vol.); 4, dioxan–acetic acid (4:1, by vol.); 5, 95% ethanol–pyridine–water (3:1:1, by vol.) with added base as indicated, calculated as the gaseous form.

	Solvent	Formate	Fluoroacetate	Acetate	Propionate	Butyrate	Valerate
1		0·78	0·84	0·84	0·88	0·90	0·92
2		0·81	0·83	0·84	0·89	0·92	0·95
3		0·75	0·81	0·81	0·85	0·90	0·92
A		0·71	0·79	0·79	0·87	0·90	0·92
4		0·31	0·70	0·54	0·77	0·85	0·86
5	+1% NH_3	0·27	0·53	0·67	0·78	0·86	0·89
	+2% NH_3	0·26	0·50	0·65	0·77	0·84	0·85
	+3% NH_3	0·25	0·45	0·64	0·74	0·83	0·85
	+5% NH_3*	0·25	0·37	0·59	0·68	0·76	0·84
	+1% CH_3NH_2	0·20	0·47	0·54	0·62	0·68	(diffuse)
	+1% $(CH_3)_2NH$	0·27	0·55	0·61	0·68	0·72	0·73
	+1% $(CH_3)_3N$	0·70	0·75	0·76	0·80	0·83	0·85

* Solvent B (see Methods).

buffer ammonia was used to produce an alkaline pH, a lower R_F value was observed for fluoroacetate than for acetate. Following this lead the solvent mixture B was developed (see under Methods), which produced satisfactory separation of formate, fluoroacetate and acetate or higher fatty acids.

Since the curious effect of ammonia cannot be due to the pH of the solution, a thorough study of power of this molecule. However, no explanation for the anomalous behaviour of formohydroxamic acid is available at present. When log $(1/R_F - 1)$ was plotted as function of n, the number of carbon atoms attached to the carboxyl group (Martin, 1950), the value for $n = 0$ fell above the straight line characteristic for the other fatty acids when ammonia, mono- or di-methylamine was used for chromatography.

Quantitative determination of hydroxamic acids after paper-chromatographic separation

Staining of the hydroxamic acids with ferric chloride in ethanol sets a lower limit of detectability of about 5 μg./spot. Such small quantities are, however, insufficient for extraction and colorimetric determination; e.g. in a Klett–Summerson colorimeter at least 0·3 μmole in a final volume of 5 ml. is required. In the search for a more sensitive method, attention was directed towards Blom's (1926) observation that hydroxylamine can be determined in fractions of a microgram by oxidation to nitrite, using iodine. In the present case this would require hydrolysis of the hydroxamic acid before the determination of the hydroxylamine (Feigl, 1954). We have, however, found that iodine oxidation is applicable to the hydroxamates themselves. The liberated nitrite is used to form a red azo dye. This reaction was first studied with crystalline benzohydroxamic acid, which gave identical calibration curves, whether tested in aqueous solution or after spotting on paper and subsequent extraction with 5% sodium acetate (see Fig. 1). Likewise, sodium acetate or fluoroacetate, when converted into the corresponding hydroxamic acids by the procedure described under Methods, and then spotted on paper and chromatographed with solvent A, gave well-reproducible, linear calibration curves, indicating that this method lends itself to accurate quantitative analysis. As is seen in Fig. 1, the lower limit of this method is 0·5 μg./ml. of final solution.

Since the chromatographic separation requires application of solvent B (see above), it was necessary to check whether the large excess of hydroxylamine, used in the preparation of hydroxamates, becomes quantitatively separated from the hydroxamates. Fortunately, it was found that the base migrates with the solvent front. However, an unforeseen difficulty arose. When filter paper was soaked in solvent B and then air-dried it showed a colorimetric blank value of considerable magnitude, which made the determination of microquantities of hydroxamic acids illusory. The blank value increased with increasing exposure of the paper to air. Apparently, autoxidation of the basic components of the solvent formed substances which were oxidized by iodine to nitrite and thus produced the red azo dye from sulphanilic acid and α-naphthylamine. However, when the paper was first soaked in solvent B, then dried and chromatographed with solvent A, containing acetic acid, the contaminating material was removed to the solvent front and the pure hydroxamates remained on the spots. Therefore, application of the iodine method to the chromatograms of hydroxamic acids requires two-dimensional chromatography, using first solvent B for separation and subsequently solvent A for purification.

With this method at hand, analyses were carried out with aqueous solutions (and with serum), containing 50 μg. each of formate, acetate and fluoroacetate per ml. After conversion into the hydroxamic acids, as described under Methods, one-tenth of the total volume was spotted on paper and submitted to two-dimensional chromatography. The final spots were located by the use of indicator samples run at the side of the paper and stained with ferric chloride. After elution and iodine oxidation, the red azo dye was formed and determined colorimetrically. The amounts, obtained by comparison with the calibration curves, were 4·7, 4·9 and 4·6 μg. respectively.

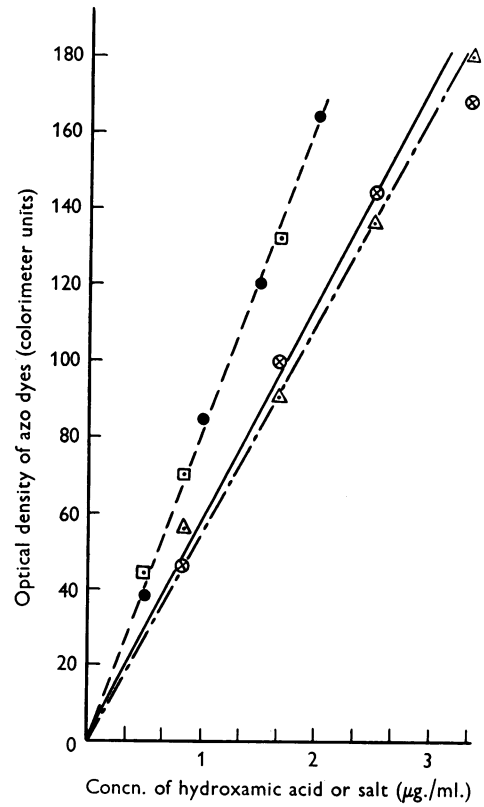


Fig. 1. Calibration curve for the colorimetric determination of hydroxamic acids by the nitrite reaction. ●, Aqueous solution of benzohydroxamic acid; ⊡, benzohydroxamic acid eluted from paper; ⊗, fluoroacetohydroxamic acid eluted after two-dimensional chromatography in solvents B and A. Concentration expressed as sodium fluoroacetate. Note the deviation from linearity with the highest value. △, Acetohydroxamic acid after two-dimensional chromatography, concentration expressed as sodium acetate.

Analysis of commercial preparations of fluoroacetate usually revealed the presence of a second substance of lower R_F value. This was identified as chloroacetate, which results probably from the synthetic method used. As shown in Table 2, chloro- and bromo-acetohydroxamic acids have practically identical R_F values and are easily separated from fluoroacetate.

The reaction between iodine and hydroxylamine is usually described by the following equation (Feigl, 1954):

$$NH_2.OH + 2I_2 + H_2O \rightarrow HNO_2 + 4HI.$$

Accordingly, each mole of hydroxamic acid should consume 4 equivalents of iodine. Direct titrations of aqueous solutions of benzohydroxamic acid with

Table 2. *R_F values of halogeno-acetohydroxamic acids*

For solvent 5, see Table 1; 6, 95% ethanol–pyridine (4:1, by vol.) with ammonia; 7, 95% ethanol with 1% of gaseous HCl.

	Solvent	Acetate	Fluoroacetate	Chloroacetate	Bromoacetate
5	+1% NH_3	0·67	0·53	0·32	0·30
	+2% NH_3	0·65	0·50	0·26	0·26
	+3% NH_3	0·64	0·45	0·24	0·23
6	+0·5% NH_3	0·51	0·28	~0	~0
	+1·5% NH_3	0·43	0·20	~0	~0
7		0·80	0·75	0·31	0·31

DISCUSSION

The present experiments show that the conditions for separation of formate and fluoroacetate from the other simple aliphatic hydroxamates are similar. Thus the method developed permits the simultaneous determination of formate and acetate, which so far met only with little success (Brown, 1950; Hall, 1950), and of acetate and fluoroacetate, which until now have resisted separation.

A new sensitive method for the quantitative determination of microquantities of hydroxamic acids has been found in the direct oxidation of them by iodine to nitrite, which then diazotizes sulphanilic acid to form an azo dyestuff with α-naphthylamine. This procedure is admirably suited for paper chromatography, since the excess of hydroxylamine is automatically separated from the hydroxamic acids.

In the nitrite method the nitrogen of the hydroxamates is the specific component which serves for colorimetric determination. Since filter paper does not contain any nitrogenous material it does not interfere with the procedure used. The advantage of this method becomes clear in the light of our efforts to determine hydroxamic acids by other means. For example, these derivatives, like hydroxylamine itself, reduce ammoniacal silver solutions, and under suitable conditions the reaction lends itself to a quantitative method for the determination of crystalline benzohydroxamic acid. However, since water or organic solvents extract from filter paper a considerable amount of reducing material, a high blank, varying with temperature and time of extraction, is obtained, which makes the silver method unsuitable for paper chromatograms.

iodine in acetic acid consumed less than half of the 'theoretical' amount. However, a similar result was obtained with crystalline hydroxylamine hydrochloride itself. The reaction mechanism is therefore still obscure. However, in a great number of analyses the new procedure has given reproducible results. Application of the method to the analysis of urine and tissue extracts will be described in a subsequent paper.

It has been observed that solvent B produces on paper substances which are converted by iodine into nitrite. Analysis of this phenomenon revealed that both ammonia and pyridine are responsible for this effect. Apparently the cellulose surface has a catalytic action on the autoxidation of these bases, which are presumably transformed into amine oxides or related products.

SUMMARY

1. Fluoroacetate can be separated from formate, acetate and other straight-chain fatty acids by paper chromatography of the hydroxamates in ammoniacal ethanol–pyridine–water.

2. The specific effect of ammonia is shared by mono- and di-methylamines, but is absent from trimethylamine. This may indicate the formation of an intramolecular hydrogen bond between an NH-group and fluorine.

3. Hydroxamic acids can be determined quantitatively by direct iodine oxidation to nitrite, which is used to form a red azo dye. The method has a lower limit of 0·5 μg./ml. of final solution.

4. The new procedure involves two-dimensional paper chromatography of the hydroxamic acids, extraction, and formation of the dye.

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The Oxidation of Tryptophan and Some Related Compounds with Persulphate

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(*Received* 30 *June* 1955)

The oxidation of tryptophan and some related compounds with persulphate has been studied in the light of recent knowledge of the metabolism of this amino acid (cf. Dalgliesh, 1951). The compounds related to tryptophan which have been oxidized include kynurenine, 3-indolylacetic acid and indole, and the oxidation of anthranilic acid has been re-examined. A number of chemical oxidations of tryptophan have been reported, and the present work the action of alkaline persulphate on DL-tryptophan, under conditions similar to those previously described for the persulphate oxidation of aromatic amines (Boyland, Manson & Sims, 1953; Boyland & Sims, 1954), has led to the formation of a number of acid-labile and other products (see Fig. 1), some of which have either been isolated or identified by means of paper chromatography.

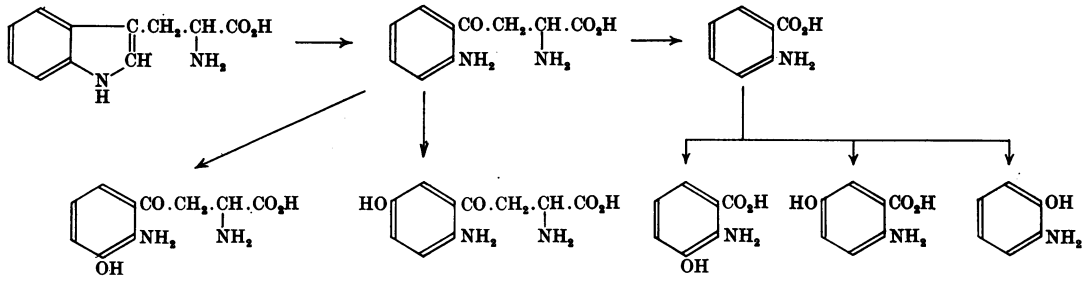

Fig. 1. Oxidation of DL-tryptophan with persulphate.

usually they involve either attack on the side chain or fission of the pyrrole ring. With ferric chloride, for example, 3-indolylaldehyde is formed (Hopkins & Cole, 1903; Ellinger, 1906), and ozonolysis yields *N'*-formylkynurenine (Witkop & Graser, 1944; Knox & Mehler, 1950). Peracetic acid, however, hydroxylates L-tryptophan in the 2-position to yield β-3-oxindolylalanine (Witkop, 1947); Dalgliesh (1954) has shown that with the ascorbic acid-hydroxylating system of Udenfriend, Clark, Axelrod & Brodie (1954) and Brodie, Axelrod, Shore & Udenfriend (1954), 5- and probably also 7-hydroxytryptophan are formed. In

EXPERIMENTAL

Melting points are uncorrected.

Paper chromatography. Descending chromatograms on Whatman no. 1 chromatography paper were developed with the organic phase of *n*-butanol–acetic acid–water (4:1:5, by vol.) (Partridge, 1946). The oxidation products were characterized on the papers by examination under a Hanovia Chromatolite ultraviolet lamp, and by spraying the papers (*a*) with 5% *p*-dimethylaminobenzaldehyde in 10% aqueous ethanol containing 2·5% HCl (Ehrlich's reagent), (*b*) with 0·4% ninhydrin in *n*-butanol saturated with water (the colours produced were sometimes slow in appearing, so that the chromatograms were examined 2 hr.