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# Biochemistry of Fluoroacetate Poisoning. Isolation of an Active Tricarboxylic Acid Fraction from Poisoned Kidney Homogenates

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The experiments described in this paper* were done in order to advance knowledge of the biochemistry of fluoroacetate poisoning. The proved failure of this compound to inhibit isolated enzymes made it difficult to relate the toxic action to an enzymic effect, until Bartlett & Barron (1947) made the important step of showing that in tissue slices fluoroacetate led to accumulations of acetate. Another significant point was made by Saunders (1947) and colleagues who found an alternation in the toxicity with increasing length of carbon chain in the $\omega$-fluoroesters; compounds with an even number of carbon atoms were toxic. Then Liébecq & Peters (1948, 1949) found an instance with centrifuged kidney homogenates where fluoroacetate caused depression of oxygen uptake without an increase of acetate but with accumulation of citrate; at the same time the oxidation of added citrate was inhibited by the poison. This led them to propose the jamming hypothesis; according to this sodium fluoroacetate can be activated and condensed with oxaloacetate to form a fluorotricarboxylic acid, which either itself, or in the form of some derivative, inhibits the tricarboxylic acid cycle and so causes accumulation of citrate. A similar hypothesis was advanced independently by Martius (1949). Kalnitsky & Barron (1948) later reported accumulation of citrate, but interpreted this differently. It was an important extension from these *in vitro* tests when Buffa & Peters (1949) found that the accumulation of citrate was also induced readily *in vivo* by injection of fluoroacetate into animals. This has been recently confirmed by Potter & Busch (1950).

The best proof for the jamming hypothesis would be the isolation of a toxic fluorotricarboxylic acid either from the tissues of poisoned animals or from the products of some poisoned tissue preparation. Calculation showed that this was impracticable *in vivo*, owing to the small amount of substance present in poisoned tissues; attempts were therefore made to isolate the tricarboxylic acids formed during the oxidation of fumarate by a kidney homogenate *in vitro* in the presence of fluoroacetate. In this work, there will be described the isolation of a 'citrate' fraction from poisoned kidney tissue, a component of which has the power to inhibit citrate metabolism *in vitro*; this is not fluoroacetate, but is believed to be a tricarboxylic acid which contains fluorine in small amount.

* The main facts recorded here were given in lectures to the Physiological Society at Oslo in March 1950, also in Uppsala; see also Buffa, Lotspeich, Peters & Wakelin (1950).

## EXPERIMENTAL

### *Determination of the inhibitory activity of tricarboxylic acid fractions using kidney homogenates*

The object of the test was to determine the capacity for inhibiting the disappearance of citrate. Two methods were used.

(1) The technique developed in this laboratory by Liébecq & Peters (1949) was slightly modified. The guinea pig kidney homogenate, made in a cooled mortar, was not squeezed

through muslin before the cold centrifugation; the duration of this was increased up to 60 min.; the solid residue so obtained was resuspended in 0·9% KCl buffered with 0·1 M-phosphate (pH 7·2) by adding the cold solution drop by drop. The suspension was filtered through muslin placed on a funnel and a glass rod was used to push the homogenate through the muslin. This gave a fine suspension which could be pipetted easily. 1·9 ml. of the enzymic homogenate (equivalent to approx. 200 mg. of kidney cortex) was added to flasks kept on ice and previously prepared with the appropriate substrates: sodium citrate (10 $\mu$mol. in 0·2 ml.); $MgCl_2$ 0·1 ml. (0·8% $MgCl_2.2H_2O$ equivalent to 100 $\mu$g. Mg); the fraction to be tested; and 0·9% KCl to 2·9 ml.; 0·1 ml. of adenosinetriphosphoric acid (ATP) (equivalent to 1 mg. of the Ba salts) was added at the end. The final phosphate concentration was 0·068 M, pH 7·2; the gas phase, air; temperature 38°. At the end of an equilibration period of 10 min. (the $O_2$ uptake being measured for 20 min.) or after a total period of 30 min., the flasks can be removed from the bath and cooled on ice; 1 ml. of 25% trichloroacetic acid (TCA) was added. After standing for 15 min. the TCA extract was filtered and the solid residue washed twice with TCA (5%); citrate estimations were done upon the combined extract and washings, using conveniently one-eighth of a bottle for each estimation.

It is to be noted that the homogenate must not be frozen at any stage, and that our Waring blender produced inactive preparations.

(2) *Simplified standard technique*, which has been used in the later experiments where no measurements of $O_2$ uptake were made. The test was conducted in ordinary 50 ml. Pyrex conical flasks which contained citrate (10 $\mu$mol.) and Mg (100 $\mu$g.) in 1 ml., the neutralized sample for test +0·9% KCl, 1 ml.; phosphate buffer to make a final concentration of 0·05 M, pH 7·2, and 2·0 ml. homogenate, equivalent to 200 mg. kidney cortex. The homogenate was prepared fresh and used without centrifuging; it was added to the flasks and their contents previously cooled in ice; a final addition of 0·1 ml. ATP, equivalent to 1 mg. Ba salt, was made. The flasks were shaken (120 per min.) for 30 min. at 38° in air. After this 1 ml. TCA (25%) was added and the procedure was as before.

Table 1. *Illustration of methods*

(Inhibition of citric acid disappearance by sodium fluoroacetate in guinea pig kidney homogenate (≡200 mg. tissue) reinforced with $Mg^{++}$ (100 $\mu$g.) and ATP (≡1 mg. Ba salt); phosphate buffer final concentration 0·05 M; pH 7·2; air; temp. 30°. Time 30 min.)

| | Citric acid ($\mu$mol.) | |
|---|---|---|
| | Control | +50 $\mu$g. fluoroacetate |
| Initial | 10 | 10 |
| After 30 min. | 4·09 | 8·75 |
| Disappeared | 5·91 | 1·25 |

Difference due to inhibitor 4·66 $\mu$mol.

The extracts were boiled with $H_2SO_4$ (0·3 ml. 50% (v/v)/tube) for 1 min. before the estimation of citrate. It should be pointed out that fluoroacetate itself inhibits the disappearance of citrate when added in relatively large amounts, presumably because it condenses with traces of oxaloacetate formed from the added citrate to form the 'active' substance.

The activity can be expressed either as the percentage inhibition ($\mu$mol.) with respect to the control, or better as the number of $\mu$mol. inhibited by the 'active' fraction. Table 1 gives an example of the effect of fluoroacetate; calculated as percentage inhibition this would be 79%, or as $\mu$mol. inhibited 4·66 $\mu$mol.

*Estimation of fluorine*

A considerable amount of preliminary work was necessary before reliable results could be obtained. Care was required (*a*) in the decomposition of the fluoro-compounds, and (*b*) in the selection of sufficiently sensitive methods of fluoride analysis. In regard to (*a*) we finally adopted the bomb method upon the advice of Prof. M. Stacey, because it was uncertain whether less drastic methods would break up C—F bonds present and also to minimize the loss of volatile fluoro-compounds. (*b*) For the analysis of the liberated fluoride, we abandoned the lead chlorofluoride method (Chapman, Heap & Saunders, 1948), even after suitable modification for small quantities (R. W. Wakelin, unpublished experiments), owing to the difficulty of dealing with the accompanying phosphate. A distillation method was adopted, which was a modification of two methods reported respectively by Yoe, Salsbury & Cole (1944) and in the Medical Research Council memorandum on Industrial Fluorosis (1949).

The final technique was as follows:

*Decomposition of the organic compounds.* The dry sample (3–30 mg.) was placed in a 2·5 ml. stainless steel (or nickel) bomb with a piece of Na weighing (approx.) 200 mg. After sealing with a gold washer, the whole was heated to 550° for 1 hr. in an electric furnace. The bomb was cooled, washed externally and dried with filter paper. After opening, both the bomb and the lid were placed in a Pyrex beaker and rapidly covered with about 20 ml. of water. The extraction was completed by boiling for a few minutes, and the carbon residue removed by filtration and washed. The fluoride was removed from the filtrate and washings (approx. 40 ml.) by steam distillation as $H_2SiF_6$; this separates it from ions such as $PO_4$, which would interfere with the subsequent titration.

*Distillation.* The apparatus shown in Fig. 1 was adopted. It was made entirely of glass, and had only one joint; no dead spaces existed and it could be used both for refluxing and distillation. With this apparatus the distillation proceeded without bumping and no interfering ions were distilled or carried into the distillate. Recoveries ranging from 80 to 100% were obtained from standard fluoride solutions containing 15–100 $\mu$g. F.

The following reagents were placed in the flask: $KIO_4$, 0·2 g.; $AgClO_4$ dissolved in $HClO_4$, 2 ml. (equiv. 2 g. $AgClO_4$); 72% $HClO_4$, 15 ml.; sample, 40 ml. approx.; glass wool, 0·2 g.

$AgClO_4$ was prepared by heating $AgNO_3$ with 72% (w/v) $HClO_4$. The $HNO_3$ was distilled off and the distillation continued until the mixture had reached a temperature of 180°. The solid residue was dissolved in 72% $HClO_4$ and the colourless solution stored at 2°. The 72% $HClO_4$ occasionally contained organic material, which was removed by distillation over $AgClO_4$. The 'lead-free' glass wool of the British Drug Houses Ltd. was found to be free from fluorine.

The ground joint was lubricated at the top with a narrow ribbon of stopcock rubber grease and the apparatus assembled. The distillate was collected in a 250 ml. conical flask marked at 180 ml. and containing approx. 20 ml.

0·025 N-NaOH. The flask was gradually heated and direct distillation carried on until the temperature of the mixture was 125°. Steam distillation was then commenced and the temperature of the mixture raised to 140° and maintained between 135 and 145°. The rate of distillation was regulated so that the 180 ml. mark was reached in 30±2 min., including the direct distillation time, usually 8–10 min. The

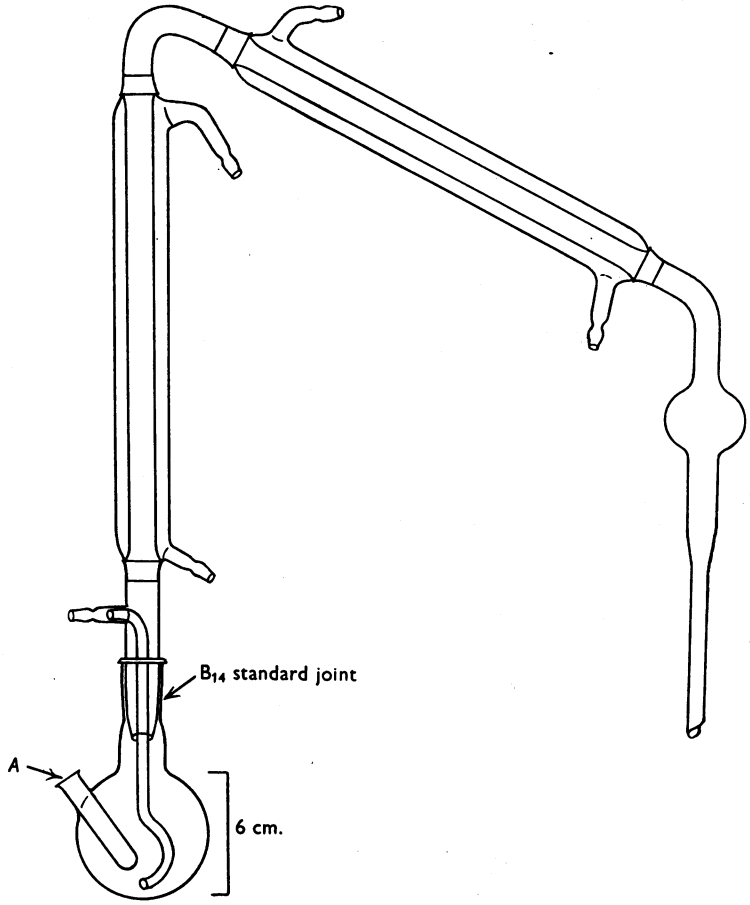


Fig. 1. Apparatus used for steam distillation in the estimation of fluoride. The thermometer is inserted into tube at *A*.

distillate was made up to 200 ml. This contained approx. 0·4 mmol. $HClO_4$. A distillation blank was run for each set of estimations and it usually gave a zero titration figure.

*Titration.* Reagents: 0·004 N-$Th(NO_3)_4$; Solochrome Brilliant Blue B.S. 0·02% in water, a solution which was renewed every 2 weeks. Chloroacetic buffer: 22·7 g. $CH_2Cl.COOH$ and 4·8 g. NaOH in 1 l.; 0·2 N-$HClO_4$; 0·2 N-NaOH. Before each set of titrations the following mixture was prepared, Solochrome Brilliant Blue B.S. 20 ml., chloroacetic acid buffer 10 ml., 0·2 N-$HClO_4$ 1·2 ml., water to 200 ml.

The titration was carried out in Nessler tubes marked at 50 ml. The use of phenolphthalein as indicator for the preliminary neutralization of the sample as recommended by some other workers in our hands sometimes gave uncertain results. Therefore phenol red was used as the indicator, and the solution to be titrated was neutralized with 0·2 N-$HClO_4$ to a yellow colour. To the samples (20 or 40 ml.) were then added the calculated amounts of $HClO_4$, 10 ml. of the buffer-indicator solution and water to 50 ml. To the blank tube was added 0·1 ml. of the Th solution; the sample was then titrated until the colour matched that of the blank. The fluorine content was calculated from a calibration curve obtained from standard fluoride solutions. Less than 1–1·2 $\mu$g. F could not be titrated and therefore no F could be detected in any original samples containing less than 6 $\mu$g.

### *Paper chromatography*

The general principles of Consden, Gordon & Martin (1944) were followed, using miscible solvents as advised by Hanes & Isherwood (1949, 1950). Many sets of solvents have been tried. Since the substances being chromatographed were often required for tests following extraction from the paper, solvents with high volatility were chosen as far as possible. Phenol red in alkaline solution was used to detect the spots. This indicator does not interfere with the citric acid estimation, and in small quantities it is known not to be toxic for the enzyme system used.

It was found that, by using the method of the ascending solvent, a good chromatogram was obtained, while with the descending solvent method the spots were less well defined with marked trailing; the former technique, therefore, was adopted.

*Paper.* Whatman no. 1 paper was used when smaller and well defined spots were desired; Whatman no. 4 was chosen when larger intervals between the spots were wanted. The preliminary washing of the paper (Hanes & Isherwood, 1949) was found to be unnecessary.

*Vessels.* In order to determine a suitable solvent set, a battery of eight cylindrical glass pots with plastic screw tops, and strips of filter paper 22 × 4 cm. were used. The solvents were placed on the bottom of the pot and the paper suspended from a string fixed across the lid. In this manner it was possible to determine in 1–3 hr. whether a given solvent set was likely to be suitable.

The final experiments were carried out in glass tanks; two paper sheets 40 × 18 cm. were suspended from two strings fixed on the glass plate covering the tank. Cylinders (2 l.) were also used as vessels.

*Procedure.* The acid solution to be chromatographed was put on the starting line with a capillary pipette and the paper was left to dry in the air. The solvent set was prepared immediately before use and placed on the bottom of the vessel; the paper was fixed on to the cover plate which was then sealed to the tank with vaseline. The size of the paper sheet and the quantity of solvent were such that the former was submerged to a depth of 2–4 mm. Most of the chromatography has been carried out at constant temperature, usually at 20°.

After development, the paper was dried in air or in a stream of warm air, and it was then sprayed with the following solution: Phenol red, 50 mg.; ethanol 96%, 20 ml.; N-NaOH, 2 ml.; water to 100 ml.

The bright yellow spots on the red background were immediately outlined with a pencil since they lasted only for a few minutes. On drying, the paper became entirely yellow; but by exposing the paper to $NH_3$, the red colour could be restored.

### *Other estimations*

Estimation of citric acid was carried out by the method of Pucher, Sherman & Vickery (1936) as modified by Buffa & Peters (1949), and of P by that of Berenblum & Chain (1938) with the adaptation worked out by Dr L. A. Stocken in this laboratory.

### *Chemicals*

These were 'Analar' unless otherwise stated. The ATP specimens used were mostly from commercial sources and when tested were about 80% pure. *cis*-Aconitic acid and DL-*iso*citric acid were prepared by Mr R. W. Wakelin.

## RESULTS

### *Methods developed for the preparation and isolation of the citric acid fraction*

*Preliminary remarks.* Upon the working hypothesis that a new F-tricarboxylic acid responsible for the jamming of the cycle would have properties similar to citric, *cis*-aconitic and D-*iso*citric acids, customary methods of isolation of citric acid with suitable modifications were applied to the trichloroacetic acid filtrates containing the citrate formed during *in vitro* experiments by the action of fluoroacetate. By the '*in vitro*' method, accumulations of citrate can be obtained seven to eight times larger than those produced *in vivo*. Before describing the final technique employed, some remarks are needed upon the steps which were tried and discarded. Removal of protein by boiling or precipitation with metaphosphoric acid was much less satisfactory than by precipitation with trichloroacetic acid. The isolation of citric acid by evaporation to a small volume, followed by acidification and extraction with ether, was unsuitable in our hands; so also was isolation as the calcium salt. For this reason the standard procedure of barium precipitation at approximately pH 8·0 was finally adopted; this was followed by removal of barium as sulphate, and precipitation with lead; the procedure of Wieland & Rosenthal (1943), who used barium and lead salts for the isolation of citric acid, has been varied in an important detail by introduction of a fractionation of the lead salts by variation in pH, somewhat similar to that used in the selective precipitation of vitamin $B_1$ by phosphotungstate (Peters, 1930; Kinnersley, O'Brien & Peters, 1933). Fumarate rather than oxaloacetate was used as substrate, since it was found that oxaloacetate tends to cause inhibitions (cf. Potter, Pardee & Lyle, 1948); it was thought, therefore, that formation of citrate went more smoothly because fumarate maintained a slow and steady supply of oxaloacetate for the condensation. Pyruvate was not added; it increases the amount of citrate formed (Liébecq & Peters, 1949), but may be expected also to increase the relative proportion of pure citrate as opposed to the postulated F-tricarboxylic acid. Citric acid was estimated as a measure of the tricarboxylic acids present in the fractions, because of its relative ease of analysis; in the equilibrium mixture of the tricarboxylic acids, 80–90% is citric acid.

*Isolation of tricarboxylic acid fraction.* Some thirty preparations have been carried out using the kidneys from two to six guinea pigs at a time, sometimes more; in the early experiments the guinea pig homogenates were centrifuged before use; latterly this has not been done. Two preparations have also been made from ox kidney. Fig. 2 shows the course of citric acid production.

The method of preparation used in the latest experiments is described. The kidneys from six guinea pigs (18 g.) were rapidly removed from the animals, cooled to 1–2°, and the cortical tissue ground gently in a porcelain mortar kept on ice. Ice-cold potassium chloride (45 ml. of 0·9%) was added during the grinding; after filtering through muslin, the mixture was made up with potassium chloride (0·9%), and enough phosphate buffer pH 7·2 to make the final concentration 0·4 M. Total

volume 132 ml. This was added to a mixture containing, in 66 ml. of potassium chloride, sodium fumarate (228 mg.), magnesium chloride (7·2 mg. Mg), ATP (equiv. to 72 mg. barium salt) and sodium fluoroacetate (72 mg.); the total volume of 198 ml. was distributed in six conical flasks (100 ml.) and incubated at 38° for 60–90 min. with shaking at 120 excursions/min. in air. (In the case of the large preparations from ox kidney, the mixture was placed in 2–3 l. spherical flasks and stirred mechanically; a stream of air was also passed.) The enzyme preparation resembled the customary preparations from brain tissue employed in this laboratory, called the pyruvate oxidase system, and also the cyclophorase more recently described by Green, Loomis & Auerbach (1948); it exhibits the usual sensitivity to freezing shown by the brain pyruvate system, but has more activity in relation to the original kidney than that of Green *et al.* (1948).

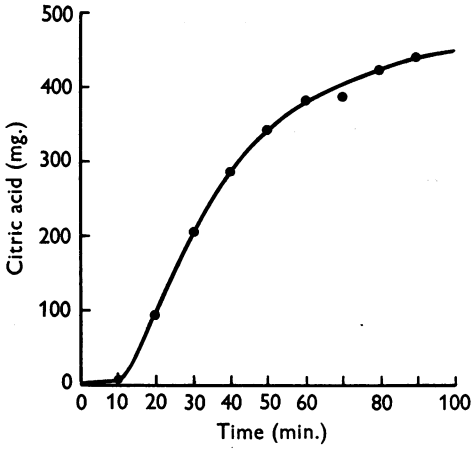


Fig. 2. Rate of citric acid production by homogenate of ox kidney at 38° by fresh tissue (≡150 g.) (centrifuged): sodium fumarate 2·80 g.; ATP≡0·44 g., Ba salt; $MgCl_2$≡880 μg. $Mg^{++}$; sodium fluoroacetate, 0·22 g.; 0·5 M-$PO_4$ buffer, pH 7·4, to a final concentration of 0·04 M; 1% KCl to final volume 1320 ml. The slow start is due to delay in reaching 38°.

The reaction was stopped by adding TCA (1 g./ml.) to a final concentration of 5%. After cooling, the protein precipitate was separated by filtration and the solid washed with 30 ml. TCA (5%). The clear yellow liquid, including the washings, was used for the subsequent fractionations.

*Fractionation of the extract*

The details of a typical fractionation will be given.

All separations were carried out by the centrifuge unless otherwise stated. The TCA extract was brought to pH 8·5 by addition of NaOH (6·6 ml., 10·0 N) and $BaCl_2$ (12·5 ml., M) added to slight excess. After stirring, the mixture was left overnight at 2–3°. The precipitate was separated and washed (twice with 15 ml. water approx.), suspended in 5 ml. water, and $HNO_3$ (40%, v/v) then added until the solution reached pH 1–2 (thymol blue). At this pH there was often a small solid residue of $Ba(NO_3)_2$ which was removed. Ba was removed from the acid extract by treatment with $H_2SO_4$ (14·0 and 1·0 N), KOH (40%, w/v) being added to keep the pH at 3·0 (bromophenol blue). The $BaSO_4$ was removed and washed with water (5 ml., twice). The supernatant, consisting mainly of tricarboxylic and phosphoric acids, was treated to complete precipitation with $Pb(NO_3)_2$ (50% (w/v), 4·5 ml.), keeping the pH at 2·0 approx. This 'acid phosphate' precipitate (I) contained mainly phosphate with small amounts of 'citrate' (7·32 mg.). It was separated and washed with 5 ml. water. The liquid phase was treated with KOH (40%) added gradually; when the pH was approx. 3·0, a gelatinous precipitate appeared. The addition of KOH and of $Pb(NO_3)_2$ was continued in small excess until the pH was 6·5 (bromocresol purple). This gelatinous precipitate II contained most of the citrate (90·5 mg.) with small amounts of phosphate; it was separated and washed twice with 5 ml. water.

Precipitates I and II were suspended in water (8 and 6 ml. respectively) and treated with $H_2S$ until the precipitation of PbS was completed (30–120 min.). The combined solutions from each precipitate, including washing with minimal volumes of water, were aerated to remove $H_2S$, and concentrated *in vacuo* to 25% of the volume, and then each made up to 10·0 ml.

A second Pb precipitation was carried out on precipitate II by bringing to pH 2·0 and treating again with $Pb(NO_3)_2$. A small precipitate (mostly phosphate) was collected (ppt. III); the pH was then taken from 2·0 to 3·0, from 3·0 to 3·5, and from 3·5 to 6·2, the respective precipitates IV, V and VI being collected. Precipitates III to VI, inclusive, were freed as above from Pb by treatment with $H_2S$ and each made up to a volume of 6·0 ml. for test. The citrate contents were III, 3·43 mg.; IV, 24·8 mg.; V, 37·8 mg.; VI, 5·13 mg. The results of tests for inhibitory activity are shown in Table 2.

Table 2. *Citric acid content and inhibitory activity of fractions isolated from kidney homogenate from six guinea pigs*

| Precipitate | Description | Citric acid content (mg.) | Activity of fraction (μmol. citric acid inhibited/mg. citric acid content) |
|---|---|---|---|
| I | Acid $PO_4$ | 7·32 | 136 |
| II | pH 2·0–6·5 | 90·5 | 67·2 |
| | Second Pb precipitation | | |
| III | Acid $PO_4$ | 3·43 | 113 |
| IV | pH 2·0–3·0 | 24·8 | 61 |
| V | pH 3·0–3·5 | 37·8 | 27·2 |
| VI | pH 3·5–6·2 | 5·13 | 0 |

The estimate of biochemical activity is made upon the basis of the number of μmol. citrate stabilized (or inhibited from disappearance) under the conditions of the test per mg. citric acid in the active fractions. On this basis it can be seen that, though the greater part of the activity is present in the precipitates obtained at less acid pH, the most active

precipitates relatively to the citrate concentration were obtained in the acid phosphate fraction.

By drying solutions like those of precipitates V and VI in a vacuum desiccator over phosphorus pentoxide at room temperature a thick yellowish syrup was obtained which could be extracted with ether and with acetone. From such ether extracts pure citric acid was crystallized of m.p. 152°, which was kindly identified as such by infrared spectrographic examination by the Ministry of Supply. This proves that the substance formed and estimated as citric acid is actually this substance.

The balance sheet from an experiment with ox kidney homogenate gives an example of the yields to be expected when working on a larger scale (Table 3).

Table 3. *Balance sheet of preparation* 24

(Ox-kidney homogenate of 21 November 1949 from 720 g. fresh tissue.)

| | Citric acid (mg.) |
|---|---|
| Initial TCA extract (6·85 l.) | 1178 |
| Protein residue | 11·9 |
| Ppt. insoluble at pH 2 | 3·2 |
| $BaSO_4$ | 47·2 |
| First $Pb_3(PO_4)_2$ ppt. | 15·2 |
| Filtrate from first lead citrate ppt. | 0 |
| First PbS ppt. | 2·1 |
| Second $Pb_3(PO_4)_2$ ppt. | 65·4 |
| Filtrate from second lead citrate ppt. | 0 |
| Second PbS ppt. | 0 |
| Ether-soluble fraction | 900·0 |
| Acetone-soluble fraction | 97·5 |
| Residue | 6·5 |
| Total | 1149·0 |

*Control preparations.* It was fundamental to show that the inhibitory activity of the 'citrate' fractions *in vitro* was not due to traces of fluoroacetate escaping the methods of isolation. Even though no fluoroacetate could be precipitated either by lead or barium salts under our conditions, it was conceivable that some might have been adsorbed by precipitates. Therefore the following blank experiment was repeated twice.

A guinea pig kidney preparation made as usual was incubated with the omission of the fluoroacetate; after 1 hr. incubation and cooling the TCA was added, followed by the amount of citric acid usually accumulating and the appropriate amount of fluoroacetate. The fractionation was carried through and the final citric fraction tested for activity. Both preparations gave an inactive product.

### *Further analysis of 'active' preparations by chromatography*

*Localization of activity in tricarboxylic acid fraction.* Preliminary trials showed that some components of the active fractions were migrating only on the alkaline side (cf. Hanes & Isherwood, 1949) and therefore alkaline sets of solvents were mainly used. By the use of propanol and ammonia-water mixtures, the 'active' fractions showed two or three spots on the chromatogram. Table 4 gives the results of one such trial of three fractions.

By comparison with the chromatogram of phosphoric and citric acid, it appeared that the spots with $R_F = 0$ were due to phosphoric acid, and the spots with $R_F = 0{\cdot}212$–$0{\cdot}214$ to tricarboxylic acids; the latter was confirmed by showing that an aqueous extract of the spot with $R_F = 0{\cdot}212$ from fraction 28-2 contained 90% of the citric acid initially present.

The migration of the 'active' fraction was then tested by the use of a double chromatogram, one half of which was used to localize the spots and the other half to test the activity. Disks of paper were cut from the second half at places corresponding to the three spots and extracted with 0·9% potassium chloride. A portion of the extracts was then used for the activity test given in column 4, Table 4. The experiment showed clearly that the active compound was not distinguished chromatographically from the tricarboxylic acids in the fraction.

Attempts were made to identify the spot with $R_F = 0{\cdot}320$ in fraction 28-2. Using the same solvent system as in Table 4, the $R_F$ value of the dicarboxylic acids succinic, fumaric and maleic acids (0·321) was very close to that of the unknown. Malonic, maleic and α-ketoglutaric acids also showed the same $R_F$ as the other dicarboxylic acids; fluoroacetic acid showed a high $R_F$. From this it follows that under these conditions, the number of carboxyl groups present in the molecule plays a more important role than their spatial configuration in determining the migration on the paper. It also follows from the

Table 4. *Chromatographic behaviour of active fractions* 28-1, 28-2 *and* 28-3

(Whatman no. 4 paper; solvents, propanol 6 vol./ammonia (sp.gr. 0·880), 3 vol./water 1 vol. Time 13 hr.: temperature 22°. Ascending solvent travel, 21·4 cm.)

| Spot no. | Fractions | | | Biochemical activity | Constituents |
|---|---|---|---|---|---|
| | 28-1 | 28-3 | 28-2 | | |
| 1 | $R_F = 0$ | $R_F = 0$ | $R_F = 0$ | Nil | Phosphoric acid |
| 2 | $R_F = 0{\cdot}214$ | $R_F = 0{\cdot}214$ | $R_F = 0{\cdot}212$ | + | Tricarboxylic acids |
| 3 | None | None | $R_F = 0{\cdot}320$ | Nil | Dicarboxylic acids |

absence of a fluoroacetate spot that fluoroacetic acid has been separated in the process of fractionation.

*Controls to establish the absence of fluoroacetate from the 'active' fractions.* It was important to make certain that the active fractions did not contain unchanged fluoroacetate carried over by adsorption on the precipitates. Using Whatman no. 4 paper, six spots were put in position: nos. 1 and 6, fluoroacetic acid (500 $\mu$g.); nos. 2 and 5, fraction 28-2 (equivalent to 335 $\mu$g. citric acid) and nos. 3 and 4, 28-2 plus fluoroacetic acid in the same quantities. The same solvents were used; and in a time of 11 hr. at 20°, the solvent travel was 24 cm.

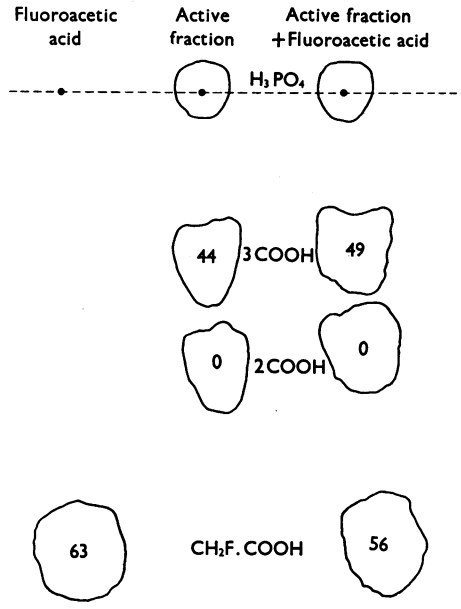


Fig. 3. Chromatograph of fluoroacetic acid, of an active fraction and of active fraction + fluoroacetic acid. The spots show the travel for fluoroacetic acid and for dicarboxylic and tricarboxylic acids. The figures placed in the spots indicate the activity (% inhibition) of the extracts of the spots. (Control recovery; for fluoroacetic acid 57%, active fraction 47%).

One half of the chromatogram (*A*), including spots 1–3, was used to localize the substances; the other (*B*) was then placed upon *A* and disks were cut from the paper in the positions corresponding to the spots. The two spots due to phosphoric acid were omitted since these had been already proved to be inactive. The disks were submerged in 3 ml. 0·9% potassium chloride for 1 hr. at room temperature and 1 hr. at 38°; 1 ml. of the extract was then used for the activity test.

To check the recovery, the same amounts of fraction 28-2 and of fluoroacetic acid used for the chromatography were placed on two disks of paper cut from sheet *B* in zones distant from the spots, the disks being immediately extracted with 3 ml. 0·9% potassium chloride.

Fig. 3 shows the chromatogram and the results of the activity test performed on the extracts of the spots, given in percentage effect upon citrate metabolism.

This experiment demonstrated clearly that: (*a*) fluoroacetic acid has an $R_F$ higher than the active fraction, (*b*) the activity was associated with the tricarboxylic acid fraction, (*c*) the activity was not due to fluoroacetate present in the fraction, since this separated quantitatively from the tricarboxylic acids.

Hence, it seems proved that an active compound inseparable from tricarboxylic acids has been formed enzymically from fluoroacetate. It must now be considered whether this is a fluoro-compound.

### *Fluorine content of 'active' fractions*

Since it was thought originally that the ratio fluorotricarboxylic acid: citric acid would be of the order 1:10 and since the material for test was scarce, in the first instance samples containing less than 6 mg. citric acid were used for the analysis. The results were all negative, when care was taken to exclude traces of phosphate from the final titration. It was then realized that it was possible that there was less fluorine present than could be detected using samples containing only 6 mg. citric acid, because in some species fluoroacetate is active in very small doses, e.g. $LD_{50}$ for the dog is 0·06 $\mu$g./kg., and at least 6 $\mu$g. fluorine must be present in the sample to be clearly detectable by the method of analysis used.

When samples of 'active' fractions equivalent to 26 mg. citric acid were taken, fluorine was found in unequivocal amounts. Table 5 shows the results upon three combined fractions, of which fraction 28-2 was the most active.

Table 5. *Analysis of fluorine content of three combined 'active' fractions*

| Fraction | $\mu$g. F/26 mg. citric acid |
|---|---|
| 28–1 | 10 |
| 28–2 | 26 |
| 28–3 | 7 |

Since these fractions still contained phosphoric acid, traces of which might interfere with the thorium titration, a control analysis was done in which 31·6 mg. orthophosphoric acid (10 mg. P) were mixed with the usual reagents and the mixture steam distilled. Even though the temperature was allowed to rise to 147° and the distillation was continued for 1 hr. the titration was negative in each of four 100 ml. portions of distillate collected. A further fluorine estimation was performed upon the inactive fraction obtained in the control preparation

previously described. This also gave a negative result. The positive result of approximately 0·1 % fluorine has also been obtained upon another very active fraction 6 months later than the results shown in Table 5. It is felt reasonable, therefore, to conclude that the active fractions contain fluorine; but they have never yet done so in amounts greater than would represent 1 % of a fluorotricarboxylic acid.

Attempts were made to prove that the active tricarboxylic acid spot upon the chromatograms contained fluorine, but the amounts of citric acid so far obtained from these spots have been too small for detection of any fluorine present.

*Comparison of biochemical activity of the 'active' fractions with that of fluoroacetate in relation to their fluorine content*

An experiment was done with a kidney homogenate in which the activity of fraction 28-2 was compared with that of fluoroacetate, both in causing the accumulation of citrate from fumarate and in this, two rats and two mice were injected intraperitoneally with large doses of 'active' fractions; none showed signs of intoxication. Both this test and the biochemical one in the preceding paragraph support the idea that the 'active' compound synthesized is not fluoroacetate itself.

*Further purification of fraction* 28-2

Attempts to purify the fraction further have been made, but have proved difficult owing to the small amount of active substance present. It is known that the 'citric acid' is accompanied by small amounts of *iso*citric acid (personal communication by Dr W. D. Lotspeich). There are usually present also as impurities phosphoric acid and small amounts of nitric acid carried over in the fractionation; neither of these are biochemically active in the kidney test. As an example, analysis of the combined fraction 28-2 showed that 1 ml. of the aqueous solution contained in addition to 26 mg. citric acid, 2·72 mg. inorganic P ($\equiv$8·6 mg. $H_3PO_4$) and 0·24 mg. organic P.

Table 6. *Comparison of the effect of active fraction* 28-2 *and fluoroacetate*

(Guinea pig kidney homogenate 1·9 ml.; ATP (1 mg. Ba salt) 0·1 ml.; $MgCl_2$ (100 $\mu$g.) 0·1 ml.; sodium fumarate (20 $\mu$mol.) or sodium citrate (10 $\mu$mol.) 0·2 ml.; active fraction or fluoroacetate (FAc) and 0·9 % KCl to total volume 3·0 ml. Citric acid accumulates from added fumarate, or added citrate is prevented from disappearing, in presence of the inhibitor.)

| | Citric acid ($\mu$mol.) | | | |
|---|---|---|---|---|
| Inhibitor ... ... | Nil | FAc | FAc | Fraction 28-2 |
| Substrate | | (0·28 $\mu$g.F) | (0·95 $\mu$g.F) | (0·26 $\mu$g.F) |
| Fumarate | 0·246 | 0·415 | 1·68 | 4·63 |
| Citrate | 0·216 | 0·216 | 0·70 | 4·64 |

inhibiting the oxidation of added citrate. Table 6 shows that the active fraction had a larger effect on the citrate metabolism, with either fumarate or citrate as substrates, even than fluoroacetate containing four times as much fluorine, and also that fluoroacetate itself had a larger effect upon citrate accumulation with fumarate than with citrate.

*Biological activity*

All the *in vivo* experiments performed with fluoroacetate showed that (*a*) it acts upon an intracellular enzymic mechanism, and (*b*) that the citric acid which accumulated inside the tissue cells did not diffuse appreciably into the extracellular fluids. In fact it has been found that in rats poisoned with fluoroacetate, the level of citrate in the blood remains low while its accumulation in the cells is high (Buffa & Peters, 1949). Furthermore, it is known that citric acid injected intravenously does not penetrate the liver, muscle or heart cells (Mårtensson, 1940). Hence it was most improbable that a highly polar molecule like that of a fluorotricarboxylic acid would be active *in vivo*. In support of

Some degree of purification was achieved by the use of ethanol after conversion to the trisodium salts. Test experiments upon citrate and phosphate mixtures kept at 0·25° showed that at 25 % ethanol concentrations (v/v) both the citrate and phosphate were in solution; increase of the ethanol concentration to 52 % gave conditions in which the citrate was still soluble, but some 92 % of the phosphate was precipitated. A careful test over a narrower range gave the optimum point as 59 % ethanol for the ratio 4 % phosphate: 100 % citrate remaining in solution. A portion of the combined fraction 28-2 was treated in this way (see chart Table 7).

The main precipitate (2) containing most of the biochemical activity was a white crystalline material giving a colourless solution of pH about 7·0. It analysed as follows: 1 mg. contained inorganic P, 0·55 $\mu$g. (equiv. to 6·8 $\mu$g. $Na_3PO_4.12H_2O$); organic P 0·095 $\mu$g.; citric acid 428 $\mu$g., equiv. to 775 $\mu$g. $C_6H_5O_7Na_3.5H_2O$. On this basis 78 % of the fraction was accounted for; of the remainder some was proved by Dr W. D. Lotspeich to be *iso*citric acid.

*cis*-Aconitic acid was absent from the active fraction. This was proved in two ways: (*a*) it was found possible to get separation of *cis*-aconitic acid by paper chromatography by using the solvent set: propan-2-ol, 6 vol./pyridine, 3 vol./water, 1 vol., e.g. citric + DL-*iso*citric acids, $R_F$ 0·155; *cis*-aconitic acid, $R_F$ 0·384. With this technique none of our combined active fractions 28-1, 28-2, or 28-3 showed the presence of *cis*-aconitic acid.

Table 7. *Purification of the combined fraction* 28-2

Combined fraction 28–2
- NaOH to pH 11·2; Ethanol; at 0·5° for 2 days
  - Ppt. 1 {all the phosphate; about 20% of the initial citrate}
- Supernatant
  - excess ethanol; at −10° for 1 day
    - Ppt. 2; about 80% of the initial citrate
- Supernatant
  - at 2° for 5 days
    - Ppt. 3; about 2% of the initial citrate
- Supernatant
  - evaporation
- Gum. Ppt. 4 (traces of citrate)

(*b*) A control experiment was set up. The flasks contained kidney tissue from two guinea pigs, 6–7 g.; sodium fumarate 256 mg.; magnesium chloride equiv. to 4 mg. $Mg^{++}$; ATP (= 40 mg. barium salt); phosphate buffer 0·5M (pH 7·2) 6 ml.; 0·9% potassium chloride to final volume of 120 ml. After the usual incubation at 38° for 1 hr. and precipitation with TCA, after 5 min. the following mixture was added, in 10 ml., 80 mg. citric acid, 16 mg. *cis*-aconitic acid and 4 mg. DL-*iso*citric acid. Fractionation was carried out on the usual lines with barium and lead salts; chromatography of the isolated fraction showed that no *cis*-aconitic acid was present in it. Hence, the method of fractionation excludes any *cis*-aconitic acid which may be present initially.

In terms of the citric acid present, fractions can show very high activity. Fig. 4 gives the details of an experiment carried out on one fraction after ethanol treatment; it shows that even the equivalent of 2 μg. citric acid in this case caused an inhibition of 0·61 μmol. In this experiment the straight-line relation between inhibition and the amount of the added fraction is evident.

Though the main biochemical activity was in precipitate no. 2 (Table 7), there was some activity in precipitate no. 4, where only traces of citric acid were present. This was the only case in which there appeared to be a discrepancy between citric acid and activity; and even this may be only apparent, because there was too little of the fraction for accurate

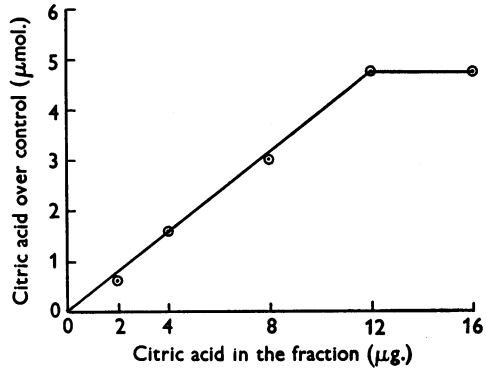


Fig. 4. Relationship between the concentration of the 'active' fraction in terms of citric acid content and the effect upon citrate disappearance in a guinea pig kidney homogenate containing initially 10 μmol. citrate and reinforced with ATP and $Mg^{++}$.

determinations. The weight of the evidence narrows down to the presence of an active fluorocitrate because of the relatively small amounts of *iso*citrate present in the fractions.

## DISCUSSION

It can be stated that an enzymically synthesized fraction can be isolated from poisoned kidney homogenates which is not fluoroacetate and which behaves like a tricarboxylic acid. Since these active fractions contain traces of fluorine which were absent from control preparations, it is inferred that a fluorotricarboxylic acid is present, which is probably fluorocitric acid. Comparative tests of the biochemical activity of the 'active' citrate and fluoroacetate show that the former is more active with either fumarate or citrate as substrates; the 'active' fraction also shows no activity *in vivo*, which is consistent with the idea that it is a tricarboxylic acid. The final decision as to the nature of the substance must await either the isolation of active fractions on a much larger scale or the synthesis of fluorocitric acid.

During the course of our work various substances have been tried as possible inhibitors of citrate metabolism; thus *trans*-aconitic acid, which might have arisen during chemical manipulations, had no activity when tried in amounts corresponding to our fractions (cf. Saffran & Prado, 1948); tricarballylic acid was also inactive. It was not realized until recently that Mårtensson (1946) had found large

increases in blood citric acid upon injecting guanidine compounds such as synthalin; we were also told by Prof. Ahlgren that in his laboratory an investigator in unpublished experiments had found inhibition of citrate disappearance *in vitro* with guanidine compounds; we can confirm that with the kidney homogenates 200 $\mu$g. synthalin B (16 $\mu$M) can cause citrate accumulation. This is an interesting fact, and further information from Lund is awaited with interest; our 'active' substance is evidently active in much smaller amounts.

The minute amount of any active fluorotricarboxylic acid which, from our analytical figures, is sufficient to affect the metabolism of citrate *in vitro* raises an interesting problem in toxicology. It is not inconsistent with the small doses of fluoroacetate known to kill some animals; the $LD_{50}$ expressed in $\mu$g. F/g. tissue for the guinea pig is 0·07 $\mu$g. approximately; this is close to the inhibitory concentration of fraction 28-2 in homogenates (0·26 $\mu$g. F/3 ml.). For the dog the $LD_{50}$ in $\mu$g. F/g. would be one-sixth of this. Furthermore, the results obtained by Hagan, Ramsey & Woodward (1950) in their estimation of fluoro organic acid-fluorine in the tissues of poisoned rats showed that the possible amount was minute. For instance they found 5 p.p.m. reckoned as fluoroacetate in the heart of the rat poisoned with 5·8 mg./kg. and killed after 5 hr. Since, according to the results of Buffa & Peters (1949), in that time the accumulation of citric acid in the heart tissue should have been of the order of 1–2 mg./g. tissue, the ratio fluorine:citric acid was from 1:1000 to 1:2000. These figures are of the same order as those analysed; for fractions 28-3, 28-1 and 28-2 the fluorine:citric ratios are 1:3710, 1:2600 and 1:1000 respectively.

The results obtained so far strengthen the evidence for the jamming hypothesis of the action of these C-F fluoro-compounds, and must be taken into account in attempts to reverse the effects of the poison *in vivo*. They make it clear why the administration of acids of the 'cycle' would be unlikely to cause improvement as these would only increase citrate accumulation.

The biological significance of these findings is particularly interesting; it has been proved that an intracellular enzyme can catalyse the formation of a substance inhibitory for another enzyme or enzymes of the same cell. Fluoroacetate differs from other toxic agents in that it is completely innocuous for isolated enzymes and becomes an effective poison only by the operation of some more organized (? mitochondrial) biochemical process. The accumulation of citrate so induced appears to upset the physiological balance of inorganic ions (Buffa & Peters, 1949; Peters, 1950). The only other analogy known to the writers is the case of pentavalent arsenicals, which become toxic when the arsenic is reduced to the trivalent form.

The mechanism by which the 'active fractions' influence citrate metabolism is not clear; it would be expected that one of the enzymes specially concerned with the citrate stage would be inhibited, that is aconitase, *iso*citric dehydrogenase, or oxalosuccinate decarboxylase. Yet, individual tests upon the isolated enzymes (Buffa *et al.* 1950) have not revealed any inhibitory action. Hence the complete enzyme system present in the kidney homogenates has properties differing from those of the isolated components. This again emphasizes the complexity of the biochemical lesion produced by this poison. It is apparently not a simple case of competitive inhibition because of the straight-line relation usually found between the amount of 'active' substance added and the effect upon citrate disappearance. It cannot be decided without further work whether the inhibition is due to blockage of an unknown enzyme or to some factor lost in the preparation of the isolated enzymes or to the organization of these enzymes in the cytoplasmic structure.

During the course of preparation of this report Elliot & Kalnitsky (1950) have reported independently the presence of fluorine in somewhat similar citric acid fractions, and Massey, who worked with Rogers (1950) upon nematodes, has stated (private communication) that he has isolated citric acid fractions with biochemical activity against citric acid.

## SUMMARY

1. The hypothesis that fluoroacetate after activation condenses with oxaloacetate to form a fluorotricarboxylic acid which then jams the tricarboxylic acid cycle has been tested by incubating homogenates of guinea pig and ox kidney cortex with fumarate, fluoroacetate and adenosine triphosphoric acid. The citric acid so formed has been isolated by selective precipitations with barium and lead at different pH values.

2. The final fractions contained citric and other tricarboxylic acids, some phosphoric acid and occasionally dicarboxylic acids; the phosphoric acid could be largely separated by precipitation with ethanol-water mixture.

3. These fractions synthesized enzymically showed *in vitro* properties similar, though different in detail, to those of fluoroacetate, in the sense that they inhibited the disappearance of citrate when added to a reinforced guinea pig kidney homogenate in the presence of either citrate or fumarate.

4. The 'active' fractions were inseparable chromatographically from the tricarboxylic acids; they contained small amounts of combined fluorine and were free from fluoroacetic acid. In terms of fluorine content, they were more active than fluoroacetate in preventing the disappearance of citrate.

5. All the evidence obtained was consistent with the conclusion that the activity of the fractions was due to the presence in them of a fluorotricarboxylic acid as postulated in the hypothesis.

Our thanks are due to Prof. M. Stacey, F.R.S., for advice upon the analysis of fluorine, to Dr C. S. Hanes and Dr F. A. Isherwood for their help in the paper chromatography. We are also grateful to Prof. T. Thunberg for drawing our attention to the results of Dr J. Mårtensson, to Prof. J. H. Burn, F.R.S., for a specimen of Synthalin B, and to Miss V. Bustin for technical assistance.

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# The Separation and Identification of Small Amounts of Mixed Amino Sugars

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The amino sugars occur as components of mucopolysaccharides, mucoids and glycoproteins. In some materials the amount and nature of the amino sugar present has been satisfactorily determined after hydrolysis of the material with strong acid, the amino sugar being isolated as the crystalline hydrochloride. In many instances, however, the yield of amino sugar is small compared with the amount estimated to be present by the colorimetric procedure of Elson & Morgan (1933) or by one of the many modifications of this method now in use (Nilsson, 1936; Palmer, Smyth & Meyer, 1937; Sørensen, 1938; Hewitt, 1938; Blix, 1948; Immers & Vasseur, 1950). A low yield of crystalline hydrochloride makes a decision as to whether the original material contains more than one kind of amino sugar difficult and the only two quantitative colorimetric methods available (cf. Elson & Morgan, 1933; Dische & Borenfreund, 1950) fail to distinguish glucosamine from chondrosamine.

An examination by means of paper chromatography of the mucoids which carry the human blood-group characters indicates the presence of two amino sugars, glucosamine and chondrosamine, in each group substance (Aminoff & Morgan, 1948; Aminoff, Morgan & Watkins, 1950). In view of the fact that a single, apparently homogeneous, mucoid had not previously been shown to contain two amino sugars it was desired to isolate crystalline derivatives of the two hexosamines.

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