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The Estimation of Serum Lipoproteins. A Micromethod Based on Zone Electrophoresis and Cholesterol Estimations

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(*Received* 29 *June* 1954)

Investigations in recent years have indicated that the lipids do not exist 'free' in serum, but are present in combination with protein; the position has been adequately summarized by Russ, Eder & Barr (1951). The development of physical methods in recent years allowed many different approaches towards elucidation of the nature of these complex serum lipoproteins.

The elegant low-temperature ethanol fractionation procedure applied to plasma by Cohn and co-workers demonstrated that almost all the plasma lipids could be accounted for in two separate protein fractions, termed IV.1 and III.0 (Gurd, Oncley, Edsall & Cohn, 1949; Oncley, Gurd & Melin, 1950). These fractions contained largely the α- and β-globulins respectively and hence the lipid fractions were termed the α- and β-lipoproteins.

A second approach to the problem was produced by the ultracentrifuge studies of Gofman, Lindgren & Elliott (1949). Subsequent comprehensive studies from this school (Lindgren, Elliott & Gofman, 1951) suggest that by this procedure the β-lipoprotein component can be shown to be a mixture of molecular species of varying densities.

A third method for the study of serum lipoproteins is zone electrophoresis as developed by Wieland & Fischer (1948), Durrum (1950), Turba & Enenkel (1950), Cremer & Tiselius (1950), Kunkel & Slater (1952). This recent, but already well-known technique, permits isolation of each electrophoretic component in a fairly pure state. Thus zone electrophoresis should be an ideal method for the study of protein–lipid relationships in serum.

Fasoli (1952) separated the plasma proteins by paper electrophoresis using the method of Flynn & de Mayo (1951) in which the paper was stained with sudan III, and showed the presence of two lipid bands corresponding with the α- and β-globulins. Roughly the same results were obtained by Rosenberg (1952) employing sudan IV as the staining reagent, while Durrum, Paul & Smith (1952) used oil red 0, and Swahn (1952, 1953) used sudan black.

Kunkel & Slater (1952) separated the lipoproteins from 2 to 5 ml. sera by zone electrophoresis in a starch block and analysed the various fractions for cholesterol and phospholipids. This method confirmed the presence of the two major lipoprotein constituents, and the results compared favourably with the lipid-staining techniques.

The purpose of the present study was to investigate the possibilities of zone electrophoresis on filter paper using about 0·1–0·2 ml. serum, followed by serial cholesterol determinations on the paper segments. The lipoprotein pattern could then be reconstructed from the cholesterol estimations. After completion of this work, a method based on the same general principle was published by Nikkila (1953), but differences exist in the electrophoresis apparatus, the technique and the micro cholesterol method used in this investigation.

MATERIALS AND METHODS

Apparatus. The supporting medium used in the electrophoresis run was Whatmann 3 MM filter paper, 14 × 5 in. The filter paper was clamped taut between two glass plates each 9 × 6 × ⅛ in. but prevented from touching the plates by four Perspex distance pieces each 6 × ¼ × ¼ in. A screw-clamping device allowed the lower glass plate to press on a heavy brass cooling plate 8 × 5 × ¼ in., which had an optically flat upper surface. A long coil of ¼ in. copper tubing was soldered to the lower surface of the cooling plate, to permit the circulation of tap water through the apparatus. The whole apparatus was then covered with an inverted glass jar.

The buffer was 0·04M diethyl barbituric acid–sodium diethyl barbiturate, pH 8·6. The Perspex electrode vessels each contained 500 ml. buffer, and the electrodes were platinum strips 4 × ½ in. The hydrostatic pressure in the vessels was equilibrated before each run by means of a glass bridge placed temporarily between the troughs.

Method. A pencil line was drawn across a piece of Whatman 3 MM, 14 × 5 in. about 4 in. from one end. Buffer was sprayed on to this line producing a damp band about 1 in. wide. Using a drawn-out capillary pipette, 0·15 ml. serum was evenly applied to the line to within ½ in. from either end. The remainder of the paper was then sprayed with the buffer solution, with the exception of about 2 in. at each end which were left dry in the meantime to facilitate the insertion of the paper in the apparatus. After clamping tightly in the apparatus with the thumb screws, the ends of the paper were dipped in the buffer solutions. At this stage, with cooling water running through the apparatus, a period of 30 min. was allowed for temperature and hydrostatic equilibration.

A potential difference of 300 v was then applied across the paper, the current carried by the paper being about 6 mA. The air temperature within the case was controlled to 12 ± 1°, the period of each run was 8 hr. Upon completion of the run, the paper was removed, and allowed to dry in air for several hours. Segments 1 cm. apart were marked out on the paper, parallel to the line of origin, and then a piece about 9 × ½ in. cut lengthwise from the centre of the filter paper, to be used later for qualitative protein detection.

Cholesterol-extraction procedure. The paper was then cut into 1 cm. pieces, combining corresponding segments from either side of the portion which had been removed for protein staining. Thus two 5 × 1 cm. pieces of filter paper were inserted into each centrifuge tube and 7 ml. acetone–ethanol (1:1, by vol.) added. The tubes were placed in a water bath at about 30° and the temperature was gradually raised until it reached 66°, where it was maintained for 10 min. and then allowed to cool to 40°.

The filter-paper strips were removed from the solvent by stainless-steel forceps and each strip was washed with a fine jet of acetone–ethanol, adding the wash liquid to the extract. The centrifuge tubes were placed in a water bath at 60° and the solvent was completely removed by a gentle stream of air. A thin glass rod was placed in each tube, and all the tubes were set in a beaker of sand in an oven at 110° for 30 min.

Cholesterol estimation. Each tube was then treated with 0·5 ml. A.R. glacial acetic acid, followed by 1·0 ml. freshly prepared ice-cold acetic anhydride–sulphuric acid reagent (20:1, by vol.). The contents of each tube were thoroughly stirred and incubated at 25° in the dark for exactly 30 min. The resultant coloured solutions were transferred by dry Pasteur pipettes to a glass cell of capacity 1 ml. and light-path 2 cm. and the optical density of each solution was read in a Spekker absorptiometer using Ilford filter no. 607 (max. transmission 580 mμ. onwards). The cholesterol contained in each sample was determined from a calibration graph prepared from standard cholesterol solutions.

Plotting the amount of cholesterol found per segment against the migration distance from the origin yields the lipoprotein pattern. A typical example of a pattern shown by normal serum is shown in Fig. 1. For comparison, the result of the qualitative protein staining from the same run is shown on the figure also. The bromophenol blue method of Durrum (1950) was used in the latter procedure.

It was shown that the presence of diethylbarbituric acid did not affect the cholesterol colour reaction, hence the small quantity of buffer eluted from the paper by the lipid solvent would not influence the subsequent cholesterol determination.

Calculation of α-:β-lipoprotein ratio. A line was drawn on each lipoprotein pattern parallel to the base-line in such a manner that both the cholesterol (lipoprotein) peaks were isolated from as much 'background' material as possible. This is shown as line A–A_1 on Fig. 1. From this datum line, the area under each component was calculated, and the cholesterol on the α-lipoprotein expressed as a percentage of the total cholesterol on both the α- and β-lipoproteins.

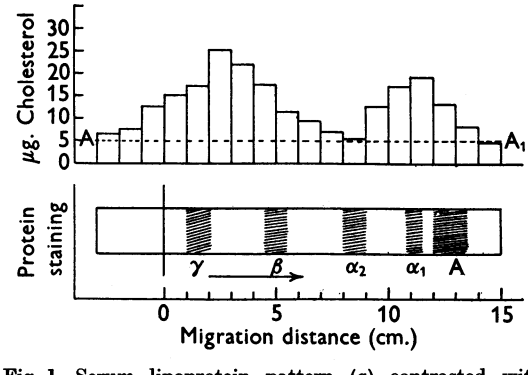

Fig. 1. Serum lipoprotein pattern (*a*) contrasted with qualitative protein staining from same paper (*b*). For details of electrophoresis and cholesterol see text. The qualitative staining for protein was done with bromophenol blue.

EXPERIMENTAL

Experiments performed to justify the procedure

The initial experiments on the separation of the lipoproteins were performed using the 'hanging paper' method of Flynn & de Mayo (1951), but serial lipoprotein results were disappointing in their reproducibility. This was thought to be due to the buffer gradient throughout the paper, because upon changing to the filter-paper technique of Kunkel & Tiselius (1951) the results became more reproducible. In the latter technique the paper is clamped horizontally between two glass plates, thus eliminating the buffer concentration gradient effect, and also the evaporation difficulties. However, while it was possible to achieve resolution of the lipoproteins by this method, a difficulty arose in that the lipoprotein component bands and the bands due to the qualitative staining of protein were more diffuse by this method than when run free in air. In an attempt to obtain the advantages of both methods, the procedure previously outlined was adopted, namely to run the filter paper horizontally without touching the glass plates. Fig. 2 shows the lipoprotein pattern obtained from a normal serum estimated by the method outlined, but *a*, run in air (Flynn & de Mayo, 1951); *b*, run between glass plates (Kunkel & Tiselius, 1951); and *c*, run horizontally in air. All were run under the same conditions of buffer ionic strength, pH, time, temperature, etc.

These results showed that the lipoprotein pattern appeared much sharper when the paper was run in air.

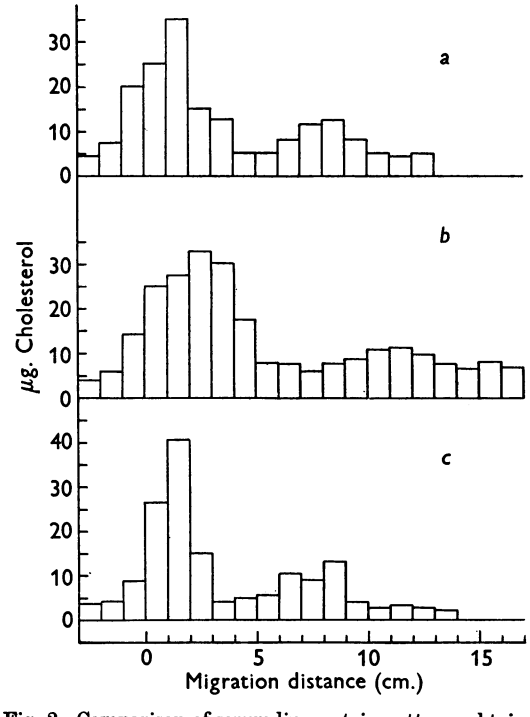

Fig. 2. Comparison of serum lipoprotein patterns obtained by electrophoresis according to: *a*, Flynn & de Mayo (1951); *b*, Kunkel & Tiselius (1951); and *c*, the present method. *a*, run in air; *b*, run between glass plates; *c*, run horizontally in air. For other details see text.

Even treatment of the glass plates with silicone grease failed to improve significantly the definition of the pattern. Nevertheless, in a series of sera run horizontally between silicone-treated glass plates, and horizontally in air, the α-:β-lipoprotein ratios were not significantly different. The sharper definition of the lipoprotein patterns obtained by the 'air gap' method decided in favour of this technique.

Ionic strength of the buffer. Under the conditions outlined in the method, with buffer of $I = 0{\cdot}10$, an electrophoresis run of 8 hr. failed to separate completely the α- and β-lipoproteins. As the ionic strength of the buffer decreased the resolution of these entities increased. However, at the lowest value tried, $I = 0{\cdot}02$, the pH of the buffer was difficult to control and for this reason the concentration found most suitable was $I = 0{\cdot}04$.

Change in buffer pH *during electrophoresis.* Employing 500 ml. buffer of $I = 0{\cdot}04$ in each vessel, the change in hydrogen-ion concentration between the anode and the cathode vessels was less than 0·05 pH unit/hr. This showed that the ionic strength of the buffer and the capacity of the vessels were adequate for the electrophoresis period used.

The effect of temperature on the lipoprotein pattern. The influence of temperature on the lipoprotein separation was studied, and it was found that there was little difference in the lipoprotein patterns obtained at 1, 8 or 12°; above this last temperature there was a steady deterioration in the resolution of the α and β fractions.

Thus for the purpose of this investigation, cold water from the mains was passed through the cooling device in order to keep the temperature within the case as constant and low as possible, i.e. $12 \pm 1°$.

Paper temperature during electrophoresis. Since the temperature recorded by the above procedure was the air temperature, it was thought advisable to study the changes in temperature (if any) which occurred along the paper and throughout a normal run of 8 hr. For this purpose a thermistor device (Standard Telephones and Cables Stantel Type U 2361/20) and galvanometer were used coupled to a temperature-calibrated Wheatstone bridge. Using the electrophoresis apparatus as detailed above the temperature variation along the paper was negligible. A slight drop in temperature at the centre of the paper was due to the sag in the paper which brought the latter closer to the cooling plate assembly. During an 8 hr. run there was no appreciable change in temperature at the centre of the paper.

Rate of migration of lipoproteins. The rate of migration of the lipoproteins had to be studied in order to define the optimum time of electrophoresis. It was found that a 6 or 8 hr. run caused effective separation of the α- and β-lipoproteins, while 12 hr. and over made the patterns diffuse. In order to obtain maximum resolution within as short a run as possible the period of 8 hr. was selected.

Comparison of the lipoprotein patterns obtained from plasma, serum, and dialysed serum. A comparison was made of the lipoprotein patterns obtained by this method, of oxalated plasma, serum, and serum dialysed for 48 hr. at 3° against 0·04M diethyl barbiturate buffer, all obtained from the same blood specimen.

The results are shown in Fig. 3. While the α-:β-lipoprotein ratios in these preparations were similar, the shape of the β-lipoprotein peak was altered. For instance, in serum there appeared to be less 'adsorption' of cholesterol near the origin, producing a more symmetrical β-lipoprotein peak with less tailing. Presumably this was due to

removal of fibrin, which tends to be deposited at the origin, possibly trapping some lipid matter.

The lipoprotein patterns of the dialysed sera samples differed little from the corresponding non-dialysed samples, and the α-:β-lipoprotein ratio was unaffected by this procedure. Thus subsequent studies were performed with non-dialysed sera.

Comparison of the lipoprotein pattern obtained with 1 cm. and 0·5 cm. segments. In order to establish whether the 1 cm. segment cuts were of a suitable size to give an accurate representation of the lipoprotein pattern the following procedure was adopted. A number of sera were run in duplicate under standard conditions, one paper being cut into 1 cm. and the other into 0·5 cm. segments. The cholesterol was eluted and estimated in the usual manner. It was found that similar lipoprotein patterns were obtained by each method. Thus there seemed to be no advantage in using the smaller segments, and in fact the disadvantage that the number of cholesterol estimations was doubled while the quantity of cholesterol per segment was halved. This experiment also settled any doubt that the lipoprotein pattern produced by the 1 cm. segment method might have had a 'fine structure' which was being obscured by the magnitude of these cuts.

Examination of various types of supporting media. Whatman nos. 1, 4 and 3 MM papers were compared, and for the purpose of this lipoprotein study, Whatman 3 MM was found to be most suitable. This paper, 5 in. wide (effective width about 4·5 in.) could easily accommodate 0·15 ml. serum as a narrow band, and the paper had little (if any) lipid-soluble matter. The lipoprotein pattern obtained on Whatman 3 MM filter paper was contrasted with the pattern obtained on the same type of paper which had been extracted for 16 hr. in a Soxhlet apparatus with ether and dried at 60° before use. The serum lipoprotein pattern was unaffected by prior ether extraction of the supporting medium; furthermore the small 'background' material present in all the estimations was not decreased by this procedure. Thus for normal use, Whatman 3 MM filter paper of 'For Chromatography' grade was used throughout without any pretreatment.

To test the efficiency of the cholesterol-extraction technique. The paper segments after cholesterol extraction in the usual manner were combined and extracted with ether in a Soxhlet apparatus for 12 hr. The extracts were taken to dryness, transferred to centrifuge tubes with acetone–ethanol, and cholesterol estimated by the micromethod as detailed previously.

In eight lipoprotein estimations the mean residual unextracted cholesterol amounted to 5·1%. Thus the 95% efficiency of the simple centrifuge-tube extraction procedure was considered quite adequate. Changing to chloroform–methanol (1:1, v/v) failed to improve the efficiency of the extraction and consequently acetone–ethanol was adopted as the solvent of choice.

Comparison of this method of lipoprotein estimation with a lipid-staining technique. Eight lipoprotein estimations were run in the usual manner, but after removal of the centre section of each paper (for qualitative protein staining) only one half was employed in the cholesterol estimations. The remaining portion of each paper was stained with sudan IV employing the method of Rosenberg (1952). Qualitatively the α- and β-lipoprotein bands on the stained paper coincided exactly with the two cholesterol peaks when the latter results were plotted on the same scale. A typical example of this is shown in Fig. 4.

Reproducibility of the lipoprotein method. In order to establish the degree of reproducibility of the method the following experiment was conducted. A sample of serum was obtained from a normal subject and twenty lipoprotein estimations were performed on this single sample over a period of 11 days. The serum was stored meanwhile in the refrigerator. The α-:β-lipoprotein ratio was calculated for

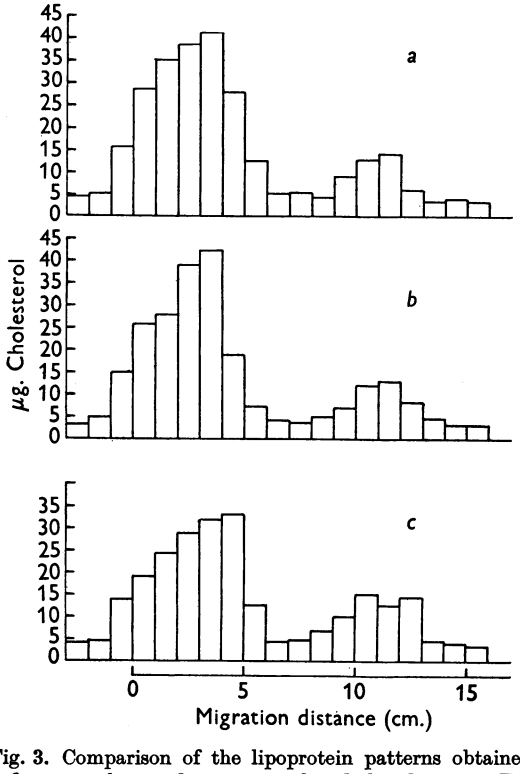

Fig. 3. Comparison of the lipoprotein patterns obtained from: *a*, plasma; *b*, serum; and *c*, dialysed serum. For further details see text and Figs. 1 and 2.

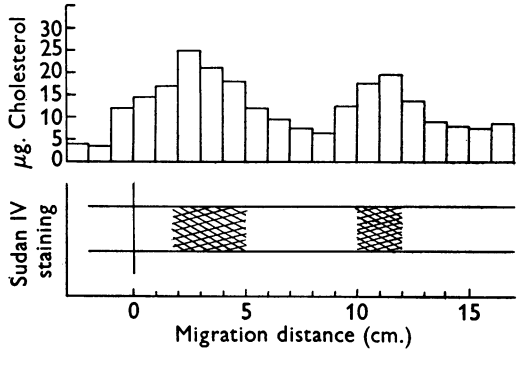

Fig. 4. Comparison of the lipoprotein pattern obtained by the method described and a pattern obtained by a sudan IV staining method.

each sample, and the mean values and standard deviations of this group were as follows:

$$\alpha:\beta::34\pm3{\cdot}6:66\pm3{\cdot}6.$$

This degree of precision appeared adequate for present purposes.

Chemical separation of the lipoproteins followed by electrophoresis of the components. As a final check on the method the α- and β-lipoproteins were separated by the Cohn fractionation procedure (Cohn *et al.* 1950). The unfractionated serum, fractions (I + II + III) and fractions (IV + V) were submitted to zone electrophoresis followed by lipoprotein estimation in the usual way. The results (Fig. 5) showed that the serum α-lipoprotein occurred in fractions (IV + V) while the β-lipoprotein appeared in fractions (I + II + III). Thus lipoprotein data obtained by this procedure should correspond with those obtained by the Cohn method.

DISCUSSION

This method for the estimation of serum α- and β-lipoproteins is relatively simple, cheap, and yields very reproducible results. Under the electrophoresis conditions described, it is possible to separate the two main lipoproteins, without separating the 'α-' lipoprotein into its main α_1 and lesser α_2 fractions. The concentration of each lipoprotein is assessed from the amount of cholesterol associated with it and this quantity is derived from the summation of serial micro cholesterol determinations. The various check experiments outlined in the text suggest that the conditions chosen for this separation approximate to the ideal.

A possible slight source of error in the calculation of the α-:β-lipoprotein ratio is due to the method of cholesterol estimation. The ratio of free cholesterol to ester cholesterol is lower in the α-lipoprotein than in the β-lipoprotein (Kunkel & Slater, 1952), and since ester cholesterol gives a more intense colour in the Lieberman–Burchardt reaction, (Gardner & Williams, 1921) this will tend to increase the 'α' component owing to its higher ester content. This small error, while interfering with the calculation of the absolute ratio of α-:β-lipoprotein, does not detract from the usefulness of the technique as a comparative method. An advantage of this micromethod for the estimation of serum lipoproteins lies in the very small quantity of blood required—a finger-prick sample would suffice.

The sera used in this study were from normal fasted subjects. The effect of chylomicra on the lipoprotein pattern, and the variations of these patterns under physiological and pathological conditions will be reported elsewhere.

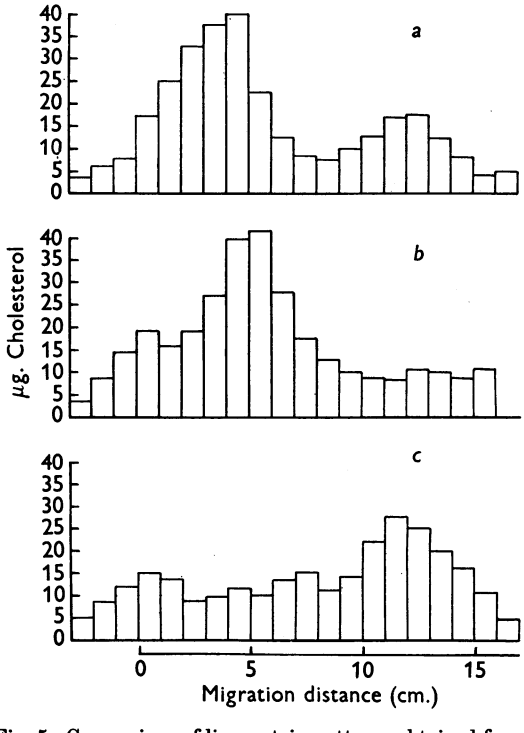

Fig. 5. Comparison of lipoprotein patterns obtained from: *a*, unfractionated serum; *b*, Cohn fraction (I + II + III) (i.e. β-lipoprotein); and *c*, Cohn fraction (IV + V) (i.e. α-lipoprotein).

SUMMARY

1. A micromethod for the separation and estimation of the serum lipoproteins is described.

2. The procedure involves filter-paper zone electrophoresis of serum, followed by segmentation of the supporting media, elution of cholesterol and estimation of the latter by a micromethod. Summation of the cholesterol data permits calculation of the ratio of α- to β-lipoproteins.

The author is indebted to Mr J. F. Syme and Mr W. C. Cooper for skilled technical assistance. This work is part of a programme of research undertaken on behalf of the Coronary Thrombosis Study Group of the Standing Medical Advisory Committee (Scotland). The author wishes to thank the Advisory Committee on Medical Research (Scotland) for a grant from which the expenses of the work were defrayed.

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The Activation of Aconitase by Ferrous Ions and Reducing Agents

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(*Received* 17 *March* 1954)

Dickman & Cloutier (1951) showed that aconitase is dependent on the presence of ferrous ions and a reducing agent for maximum activity. Qualitative studies indicated that the ferrous ion is capable of forming complexes with the substrates of aconitase and with the enzyme under the conditions of optimum enzyme activity. The authors explained the action of ferrous ion in terms of the hypothesis advanced by Smith (1951), namely, that the metal is responsible for the linkage between the apoenzyme and the substrate in the aconitase–tricarboxylic acid complex. They assumed that the reducing agents played a double role in maintaining the iron in the ferrous state and in keeping the reducing groups of the protein in the reduced state.

The high residual activity of the aconitase preparation of Dickman & Cloutier (1951) in the absence of ferrous ion and reducing agents did not permit of a more detailed investigation of the function of these substances in the aconitase system. The aconitase preparation obtained from pig heart by the method of Morrison (1954*a*) provided a means of doing this. The preparation showed little activity in the absence of ferrous ion and a reducing agent, whereas it showed considerable activity on the addition of these substances. This preparation was used for testing the effect of ferrous ion and reducing agents on the aconitase activity. The results reported in this paper are consistent with the idea that both ferrous ion and the reducing agents act as activators or prosthetic groups of aconitase and are thus concerned in the formation of the active enzyme complex. It is suggested that the active form of the enzyme may be an enzyme–Fe^{2+}–reducing agent or an enzyme–Fe^{2+}–activator complex. For a preliminary communication, see Morrison (1954*b*).

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EXPERIMENTAL

Methods

Details of the methods for the preparation and activation of aconitase as well as the determination of enzymic activity have been previously described (Morrison, 1954*a*). Electrophoretic analysis of the final enzyme preparation showed that the major component, which possessed aconitase activity, formed approximately 75% of the total protein present. After dialysis against 0·004M citrate buffer, pH 5·7, the enzyme could be stored in the frozen state for many weeks with only slight loss of activity. After activation with 5×10^{-4}M-Fe^{2+} and 10^{-2}M cysteine, 1 mg. of this preparation was capable of forming 230 μmoles of citric acid from *cis*-aconitic acid in 15 min. at 30° and pH 7·4 in the absence of buffer.

Aconitase was activated by adding a sample of the enzyme to a solution containing the indicated amounts of ferrous ammonium sulphate and cysteine, or other reducing agent. The mixture was then neutralized to pH 7·4 with N-NaOH, using a glass electrode and, except where otherwise stated, was incubated in an ice bath for 1 hr. before the determination of aconitase activity. Activation could not be carried out in the presence of a buffer on account of the ability of buffers to inactivate aconitase in the absence of substrate (Morrison, 1954*a*).

The conditions used for the determination of aconitase activity are described in the legends to the figures. Buffers were used in the later experiments for it was found that when the enzyme was added to a mixture of substrate and buffer, there was no loss of enzyme activity over the period of the test.