The Estimation of Serum Proteins by Electrophoresis on Filter Paper

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The publication of a method for separation of proteins by electrophoresis on filter paper (von Turba & Enenkel, 1950) has led to widespread application, and already many reports have appeared of its use in qualitative analysis.

Doubt has been cast on the quantitative applicability of the method (Slater & Kunkel, 1953), but evidence will be presented showing that results can be obtained, accurate for any component of a mixture to within 6% of the total protein present.

EXPERIMENTAL

Method

Three types of apparatus have been described; all three aim at minimizing evaporation from the paper surface during the separation by enclosing the paper in an atmosphere saturated with water vapour.

Glass plates (Cremer & Tiselius, 1950; Kunkel & Tiselius, 1951). The paper strip is clamped between two glass plates treated with silicone. This is technically the most difficult method, but the only one by which mobilities can be measured. In this method the spots or bands of protein often become rather diffuse.

The 'Gallows' method (Durrum, 1950; Flynn & de Mayo, 1951). The paper is suspended at the mid-point over a raised glass rod. This method gives satisfactory separations; care is needed to avoid the production of an artifact due to a pool of protein solution collecting between the paper and the rod over which it lies.

The box type of apparatus (Grassman, Hannig & Knedel, 1951). This is the type that has been used in the ensuing experiments (Fig. 1). The boxes are of Perspex, 9 × 5 × 2 in., a small flange is attached at each end to hold the paper taut, and the bottom of the box is lined with lint soaked in distilled water. The boxes are arranged in pairs bridging troughs containing buffer; the fluid levels in the troughs are kept equal by connexions of rubber tubing, in which are incorporated short lengths of 1 mm. glass capillary (Fig. 1). The two end troughs connect through a buffer-agar (1%) bridge to electrode vessels filled with buffer and containing carbon electrodes. These are connected to a source of e.m.f., controllable up to 250 v d.c.

If the protein is applied at the cathode end of the paper, then evaporation of buffer from the paper during the run leads to a continual flow of buffer towards the centre of the paper, which carries the protein forward from the origin. The endosmotic flow, which carries the γ-globulin behind the starting point in other methods, has a smaller effect so that all the fractions advance beyond the origin (Fig. 2); this is occasionally of advantage in excluding the presence in the sample of denatured protein which remains at the origin.

Technique

The filter paper (Whatman no. 3MM or no. 100) is cut into strips measuring 6 × 19 cm. A transverse line is drawn about 12 cm. from one end and five pencil marks are made on this line at 0·5 cm. intervals over the middle 2 cm. The paper is soaked in barbitone buffer pH 8·6, $I/2 = 0{\cdot}05$ (0·1 M sodium diethyl barbiturate, 0·02 M diethyl barbituric acid), and laid on filter paper to remove any excess. Five spots of 0·01 ml. of protein solution, total concentration not more than 1 g./100 ml., are put on each pencil mark with a pipette. The strips are placed in the boxes with the protein about 5 cm. from the cathode end, and the two free ends arranged to dip into the buffer troughs. With the apparatus illustrated six strips are run concurrently.

A current of 1·5 mA is passed through each strip for 18 hr. (approx. 100 v/strip). In albumin-containing solutions a trace of bromophenol blue may be added; this will bind to the albumin and act as a marker of the length of run achieved. After separation the strips are dried in an oven at

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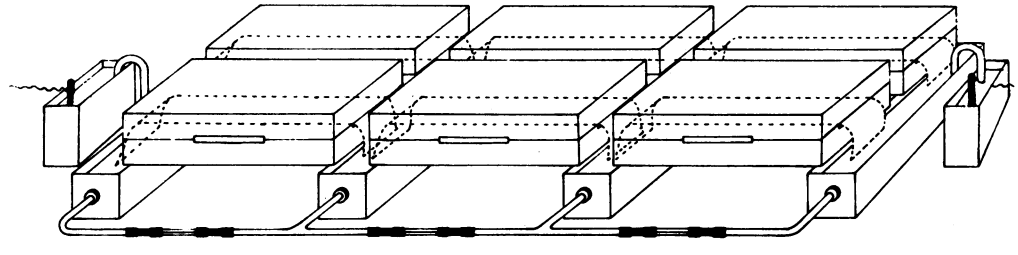

Fig. 1. Apparatus used for electrophoresis of serum on filter paper.

100°, pinned on a frame with glass pins, fixed and stained in ethanol saturated with mercuric chloride (33 g./100 ml., w/v) containing 0·1 % bromophenol blue. After 30 min. the papers are washed in 0·5 % acetic acid in water until the background is clear (four washes over 40 min.), and finally dried in the oven at 100°.

Total proteins are estimated by the biuret method (Wolfson, Cohn, Calvery & Ichiba, 1948), using a serum-albumin standard. For differential determination the strips are cut up in segments of measured width, each segment incorporating one of the visible protein fractions, or the clear area between two such fractions. Blank samples of 10 mm. width are taken at the ends of the paper, over which no protein has run (Fig. 3). Each segment is eluted with 7 ml. of 2 % (w/v) Na_2CO_3 in 50 % methanol (v/v). Each eluate is compared with elution fluid in a Hilger Spekker Absorptiometer with a filter giving maximal transmission at 6000 Å. The appropriate blank value, in respect of the segment size, is subtracted from each eluate, together with the allowance for albumin 'tail' (see below). The content of each fraction is then worked out as a percentage of the total density, any segments from between two adjacent fractions being divided between them in proportion to their respective densities.

Preliminary experiments with 'scanning' of cleared strips in a photo-electric colorimeter confirmed the observation of Crook, Harris & Warren (1952) that densities obtained are not linearly related to the quantity of protein applied; no further investigation of this method has been pursued.

In clinical work separation of serum has been preferred to plasma, since the fibrinogen confuses the electrophoretic separation of β- and γ-globulins.

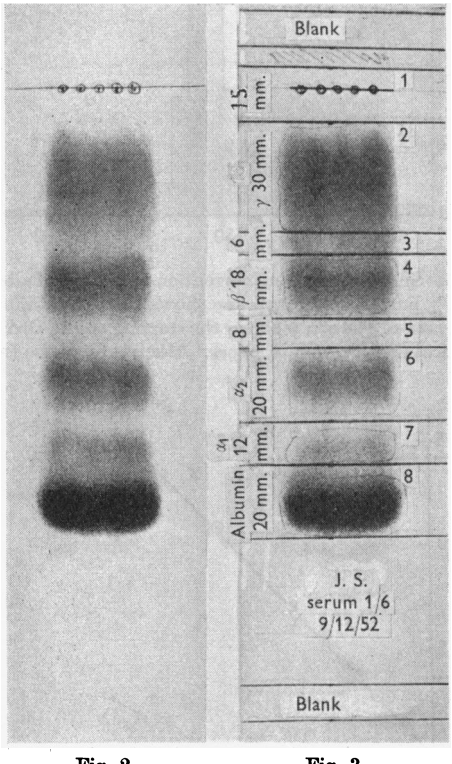

Fig. 2. Fig. 3.

Fig. 2. Separation of 0·05 ml. of serum (6·5 g. protein/100 ml.) diluted ×6 in barbitone buffer. The protein was applied in five drops of 0·01 ml. to the five spots marked on the paper; run at 1·5 mA for 16 hr.

Fig. 3. Strip shown in Fig. 2 marked out for elution.

RESULTS

Control experiments

In developing this method, rigid standardization has been necessary to obtain quantitative results. With the technique described, reproducibility and recovery is within ± 6 % of the total protein or density involved.

Representative experiments investigating a number of critical variables are described.

Concentration of dye and time of staining. Only bromophenol blue has been used, since this dye is readily and completely eluted from the paper. Maximum staining is obtained within 20 min. of application. There was little increase in density obtained when solutions containing 0·1, 1·0 and 2·5 % dye were used for staining. Quantitative results were satisfactory with the most dilute preparation.

Washing solutions. (i) Birmingham tap water, pH of 7·0, elutes the dye steadily from the paper and there is a strong possibility of uneven washing occurring. (ii) Ethanolic solutions rapidly elute a large proportion of the dye from the protein fractions. The rate of elution is equal for albumin and γ-globulin. The method is expensive, and unsatisfactory in that maximum staining is not obtained. (iii) In 0·5 % acetic acid no appreciable amount of dye was lost from the protein when samples washed 10 min., 30 min. and 24 hr. were compared. This method therefore gives maximum dye density/unit concentration of protein, and is the method of choice.

Reproducibility of staining. Absolute reproducibility of staining for a given protein concentration from day to day has not been obtained, though the proportionality of staining between fractions in a mixture of proteins remains the same. In strips run concurrently and stained together, staining is more consistently related to concentration.

Optimum concentrations of protein solution were placed on the paper, fixed and stained, and optical density plotted against protein concentration. By putting on more of a less concentrated solution of human serum albumin, estimated to be about 90 % pure by U-tube electrophoresis, it was possible to obtain larger-sized spots. Fig. 4 shows the result of such an experiment; gross understaining of protein occurs if the concentration on the paper is too great (probably due to the fixed coagulum becoming impermeable to the dye). Even with spots of an area of 11 cm.^{2} the protein concentration cannot be taken much above 0·05 ml. of a 0·8 % solution.

When the protein is run electrophoretically before staining, slightly better results are obtained, and with a spot area of 10 cm.², the density is linear up to 0·05 ml. of a 1·1 g./100 ml. solution. This understaining or 'packing' is liable to occur if the concentration of any one fraction exceeds 1·0 g./100 ml.

As a result of using dilute protein solutions, the staining of small components (e.g. α_1-globulin) becomes very faint; by putting up a line of $5 \times 0{\cdot}01$ ml., even bands of separation of adequate intensity for visual examination and quantitative estimation are obtained. Applying the protein as a hair line with a paint brush is particularly liable to produce 'packing' effects, and is not therefore recommended.

Light absorption of bromophenol blue colour with the filter used was linear with concentration to an optical density of 1·8.

Elution of the dye with sodium carbonate gives complete extraction, and the colour is stable for 24 hr. The dye fades rapidly in 0·01 N sodium hydroxide.

Type of paper. With the thinner papers (Whatman nos. 1, 2 and 4) very sharp bands of separation, particularly suitable for qualitative analysis, are obtained. 'Packing' effects however occur at very low protein concentration. Whatman no. 3 MM and no. 100 have been found satisfactory for quantitative estimation.

'Tailing'. To check whether any 'tailing' of protein occurred on the paper, a number of strips were run containing albumin marked by bromophenol blue; the blue spot was cut out while still moist, and eluted from the papers in buffer. This albumin was pure in that it had already once travelled over the paper; on running again, however, it was again found to leave a 'tail'. This tail was less on Whatman no. 3 MM than on no. 31 or no. 100 papers, and amounted to an optical density of 0·001/mm. of paper passed, irrespective of the amount of protein applied; if the solution were sufficiently dilute, then all the albumin was left in the tail and no spot appeared. As a further check, a preparation of albumin (Lister transfusion) marked with bromophenol blue was run for 16 hr., the distance migrated was noted, and the current reversed until the albumin had returned half-way to the origin; staining and elution revealed that the albumin had left some protein behind on its return passage over the paper (Fig. 5). There is little doubt that other fractions leave a similar tail, but sufficiently pure preparations have not been obtained to estimate these quantitatively; allowance is made for the albumin tail by adding 0·001 to the density of albumin for every mm. of travel, and subtracting the equivalent amount from each globulin fraction. The correction is particularly important for albumin, since it may represent an increase of 5–10 % in the estimated amounts of other components.

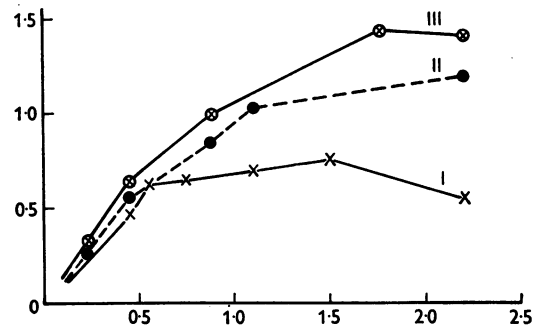

Fig. 4. Effect of spot area on staining. Ordinate, optical density; abscissa, protein g./100 ml. I, area = 4 cm.²; II, area = 7 cm.²; III, area = 11 cm.²

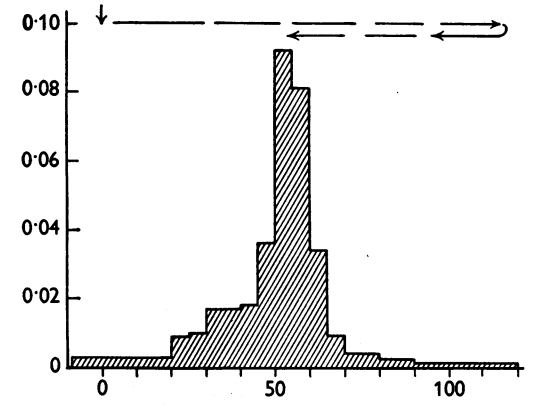

Fig. 5. Effect of reversing current on migration of albumin (90 % pure). The broken line shows the path of albumin migration. The arrow shows the starting point. Ordinate, optical density/mm. of paper. Abscissa, distance (mm.).

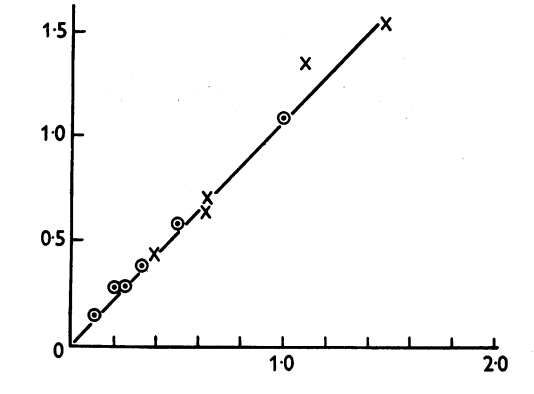

Fig. 6. Dye-binding capacity of albumin and 'cold insoluble globulin'. ×, Albumin; ⊙, cold insoluble globulin. In each case three drops of 0·005 ml. of solution were applied. Ordinate, optical density. Abscissa, g. protein/100 ml.

Table 1. *Recoveries of albumin and globulin from mixtures*

	Albumin (ml.)	Globulin (ml.)	Found (%)				Expected (%)			
			Albumin	α_2	β	γ	Albumin	α_2	β	γ
I	1·0	0	90	—	10	—	—	—	—	—
II	0·8	0·2	64·0	4·8	3·7	27·5	69·5	1·3	7·3	21·9
III	0·6	0·4	51·4	8·7	4·9	34·8	52·0	2·5	5·1	40·4
IV	0·4	0·6	32·3	4·5	5·7	57·6	37·2	3·4	3·2	56·2
V	0·2	0·8	21·2	5·5	0	73·3	24·2	4·3	1·5	70·0
VI	0	1·0	13·0	5·0	0	82·0	—	—	—	—

In each case five 0·01 ml. portions of mixture were applied and run at 1·5 mA for 16 hr. Albumin solution = 1·1 g./100 ml. Globulin solution = 1·6 g./100 ml.

Table 2. *Recovery of albumin from serum*

Case B.W. (i) Serum protein 6·3 g./100 ml. diluted × 3 (2·1 g./100 ml.), five 0·005 ml. spots. (ii) Albumin (Lister 100% electrophoretically) (1·06 g./100 ml.), five 0·005 ml. spots. (iii) Mixture 1 vol. (i) + 1 vol. (ii).

	(i) Serum (g./100 ml.)	(ii) Albumin (g./100 ml.)	(iii) Found (g./100 ml.)	(iv) Expected (g./100 ml.)	Deviation % total
Albumin	1·09	1·06	2·13	2·15	−0·5
α_1	0·12	—	0·13	0·12	+0·5
α_2	0·24	—	0·25	0·24	+0·5
β	0·19	—	0·29	0·19	+3·2
γ	0·45	—	0·35	0·45	−3·2
			3·15	3·15	

Table 3. *Recovery of fractions from mixtures of serum and urine*

	(i) Serum (g./100 ml.)	(ii) Urine (g./100 ml.)	(iii) Found (g./100 ml.)	(iv) Expected (g./100 ml.)	Deviation % total
Case K.W. (i) Serum diluted × 2 (2·1 g./100 ml.). (ii) Urine (0·84 g./100 ml.). (iii) Mixture 1 vol. (i) + 1 vol. (ii).					
Albumin	0·67	0·72	0·60	0·70	−6·8
α_1	0·09	0·05	0·06	0·07	−0·75
α_2	0·71	0	0·33	0·35	−1·4
β	0·38	0·04	0·26	0·21	+3·4
γ	0·25	0·03	0·22	0·14	+5·4
			1·47	1·47	
Case M.F. (i) Serum diluted × 1·5 (1·8 g./100 ml.). (ii) Urine (1·6 g./100 ml.). (iii) Mixture 1 vol. (i) + 1 vol. (ii).					
Albumin	0·47	0·88	0·79	0·68	+6·5
α_1	0·10	0·23	0·12	0·16	−2·5
α_2	0·54	0·15	0·31	0·35	−2·5
β	0·48	0·20	0·39	0·34	+3
γ	0·21	0·14	0·09	0·17	−4·7
			1·70	1·70	
Case J.R. (i) Serum diluted × 2 (2·2 g./100 ml.). (ii) Urine diluted × 2 (0·99 g./100 ml.). (iii) Mixture 1 vol. (i) + 1 vol. (ii).					
Albumin	0·24	0·53	0·37	0·38	−0·5
α_1	0·07	0·14	0·10	0·11	−0·5
α_2	1·07	0·07	0·58	0·57	+0·5
β	0·45	0·14	0·28	0·30	−1·2
γ	0·37	0·11	0·27	0·24	+1·9
			1·60	1·60	

In each case five 0·005 ml. portions of protein solution were applied to the paper.

Table 4. *Reproducibility of duplicate estimations at various dilutions of impure albumin*

Concentration of protein (g./100 ml.)	Optical density		Albumin (%)	β-Globulin (%)
	Albumin	β-Globulin		
1·1	1·423	0·139	91·0	9·0
	1·453	0·142	91·0	9·0
0·55	0·922	0·073	92·7	7·3
	0·817	0·078	91·3	8·7
0·275	0·317	0·020	94·0	6·0
	0·383	0·043	90·0	10·0

In each sample five 0·005 ml. portions were applied to the strip.

Dye binding capacities of proteins. It has been suggested (Kunkel & Tiselius, 1951) that the dye-binding capacities of the protein fractions differ. To investigate this, the dye intensity was compared with protein concentration, determined by biuret analysis, for albumin, γ-globulin and 'cold insoluble globulin' (fraction I of Cohn) (Morrison, Edsall & Miller, 1948). Fig. 6 shows that for equivalent amounts of albumin and fraction I, the density is directly proportional to protein concentration. In Table 1 the recoveries from mixtures of serum albumin and serum globulin are shown to be within 6 % of the expected values. Therefore for albumin, γ-globulin and fraction I, no differences in dye-binding capacity have been found.

Recovery experiments

Some examples of recovery experiments are shown. (i) With albumin added to serum (Table 2); (ii) with mixtures of serum and urine (Table 3).

Since this method was being used to compare the relative serum and urine protein concentrations in patients with proteinuria, it was necessary to determine the accuracy of recovery in these different fluids. Serum and urine proteins were therefore estimated separately and after mixture in various proportions. If any differences in binding capacity existed between the protein fractions, it should be apparent in these mixtures, since urine was relatively rich in albumin and serum relatively poor.

The errors in these estimations are closely comparable with the error in reproducibility of repeated estimations on the same serum, which was found to be ± 5 % of the total density on the paper (or of the total protein). There is thus an error approaching 100 % in the absolute estimation of a fraction comprising only 5 % of the total protein.

The reproducibility and sensitivity of the method is shown in the estimation of dilutions of a transfusion albumin preparation which contained about 10 % of a β-globulin (Table 4). By Tiselius U-tube estimation this preparation was 88 % albumin.

In this experiment a contaminating fraction was visible when a total of only 0·138 mg. protein was applied to the paper, and only 0·014 mg. of the impurity.

CONCLUSIONS

The evidence presented suggests that the method of filter-paper electrophoresis here described is quantitatively reliable when conditions are rigidly standardized. Many comparisons have been drawn between paper electrophoresis and 'classical' separation (Tiselius, 1937), some of which seem unsound in view of a number of fundamental differences in the two methods.

In the classical method the separation is carried out in buffer solution in a U-tube, and only partial separation of the components occurs, whereas in the paper method complete separation occurs (Fig. 2). The principle on which the concentrations of the various components are calculated also differs in the two methods.

The paper-strip method, as has been described, depends on dye-binding, which is directly proportional to relative protein concentrations under suitable conditions, and from the dye intensity the content of the various fractions can be calculated as a percentage of the total.

The U-tube method, on the other hand, depends on refractive index changes between the separated boundaries; this property is not specific for proteins, but depends also on lipid and carbohydrate in the protein components. For purified protein solutions the specific refractive increment can be determined, but in serum the composition of individual fractions is known to vary considerably (Longsworth, Shedlovsky & MacInnes, 1939) (e.g. the lipid content of the β-globulins in the nephrotic syndrome). Therefore estimation of protein concentrations of such sera when obtained by classical electrophoresis and based on nitrogen analysis may be considerably in error. The reproducibility and accuracy of the U-tube technique has been estimated at $\pm 1{\cdot}5$ % of the total protein (Armstrong, 1950). Evidence presented above suggests that the paper technique can give results to ± 6 % of the total protein present.

Thus, while the separation of pure proteins by electrophoresis on filter paper is not so accurate as is the 'classical' method, and it is not easily possible to measure absolute mobilities, yet it has quantitative advantages for mixtures of lipid and non-lipid containing proteins such as occur in serum, and moreover it is rapid, simple and relatively inexpensive to perform.

SUMMARY

1. A method is described for quantitative electrophoresis of proteins on filter paper, modified from that of Turba & Enenkel (1950).

2. A number of variables inherent in the method have been critically examined, including staining and washing of the papers; reproducibility of staining and optimum concentration of protein solutions; type of paper; 'tailing' of proteins on the paper during migration; dye-binding capacity of different protein fractions.

3. By recovery experiments and duplicate determinations, the quantitative accuracy of the method as described is found to be ± 6 % of the total protein present.

4. The comparative merits in the analysis of human sera of the paper method and the 'classical' Tiselius method are discussed. Apart from the

obvious advantages of simplicity and rapidity, electrophoresis on filter paper may be quantitatively more accurate in the analysis of pathological sera containing high proportions of lipid or carbohydrate.

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Time-Course of Injected Acetate in Normal and Depancreatized Dogs

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Acetic acid plays a very important role in intermediary metabolism both as degradation product of large molecules and as building block in numerous synthetic reactions.

It has been shown, mainly with the aid of isotopes, that acetic acid takes part in the syntheses of acetoacetate (Swendseid, Barnes, Hemingway & Nier, 1942), cholesterol (Bloch & Rittenberg, 1942), higher fatty acids (Rittenberg & Bloch, 1944, 1945), citrate (Stern & Ochoa, 1949), etc. Owing to the lack of specific chemical methods for the estimation of acetic acid, few studies of the metabolism of this compound have been made in which the amount of acetic acid metabolized was determined directly.

Recently Ciaranfi & Fonnesu (1952) have described a photometric micromethod for the estimation of acetic acid in blood and tissues and this procedure is suitable for such a type of investigation. With this method it has been shown that acetate is a normal constituent of blood and organs (Ciaranfi & Fonnesu, 1952), of lymph (Fonnesu & Ciaranfi, 1952) and of bile (Fonnesu, 1953*a*). Blood acetate originates either from the tissues or, by absorption, from the intestinal lumen. Skeletal muscle rapidly utilizes acetate conveyed by the blood, since the concentration of acetate in the arterial blood is greater than that in the veins draining the limbs (Fonnesu, 1951, 1953*a*; Fonnesu & Ciaranfi, 1952).

The concentration of acetate in the blood and tissues is relatively low probably because of the rapid turnover of the compound. Experiments with labelled acetate have shown that considerable amounts of acetate are transformed into other compounds and oxidized to carbon dioxide by the intact animal (Buchanan, Hastings & Nesbett, 1943; Lifson, Lorber, Sakami & Wood, 1948; Gould, Sinex, Rosenberg, Solomon & Hastings, 1949; Villee & Hastings, 1949; Pihl, Bloch & Anker, 1950; Hevesy, Ruyssen & Beeckmans, 1951; Coniglio, Anderson & Robinson, 1952; Hutchens & van Bruggen, 1952; van Bruggen, Claycomb, Hutchens & West, 1952). Significant quantities of acetate can also be oxidized by the eviscerated animal (Pihl *et al.* 1950; Wick & Drury, 1952). It has been observed that at least two extrahepatic tissues, that is, the heart (Pearson, Hastings & Bunting, 1949) and the diaphragm (Villee & Hastings, 1949) are capable of oxidizing acetate to carbon dioxide *in vitro*.

However, free acetate is known to be a metabolically inert substance and in order to react it requires activation. The work of Lynen & Reichert (1951) and of Lynen, Reichert & Rueff (1951) made it clear that 'active acetate' is in fact acetyl coenzyme A (Ac ~ CoA).

It is probable that free acetate is first converted into Ac ~ CoA. If this is true, the inhibition of such

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