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The Isolation of Albumin from Blood Serum or Plasma by means of Organic Solvents

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It has been known for some time that the trichloroacetic acid precipitates of certain proteins dissolve in methanol or ethanol whereas those of other proteins do not. Thus Delaville (1954), having shown that the trichloroacetic precipitate of albumin was soluble in alcohol, but that those of the globulins were alcohol-insoluble, utilized this distinguishing feature for the analysis of serum. He attributed the solubilizing action to some special feature of trichloroacetic acid coupled with its ability to form a very strong bond with albumin.

Several authors (Korner & Debro, 1956; Schwert, 1957; Kallee, Lohss & Oppermann, 1957) have subsequently investigated the properties of albumin after treatment with trichloroacetic acid and alcohol or acetone and have arrived at the

conclusion that the albumin, after recovery, is still in the native state, as judged by its physico-chemical properties and by its unchanged antigenic properties, but that it has acquired a slightly changed susceptibility towards proteolytic enzymes (Ram & Maurer, 1958).

A more detailed study of the behaviour of the main blood proteins and some other proteins has now shown that the solubilizing action in suitable organic solvents, on albumin, but not on globulins, is shared by a number of other acids. Some acids were found to be unable to prevent damage to the albumin in the organic solvents, whereas others preserved the albumin in a condition that is more nearly related to the native state.

This has enabled a process of separation of blood

proteins to be developed which gives albumin of a high grade of purity, in high yields.

A short account of this work has been presented (Michael, 1958).

EXPERIMENTAL

Materials. Animal serum was prepared from fresh ox and pig blood and clarified through a filter of the Seitz type. Human serum was prepared similarly. Animal plasma was prepared with citrate or oxalate as the anticoagulant. Human plasma was prepared from citrated blood. The plasma was in every case passed through a clarifying filter.

The proteins used were: porcine and bovine serum albumin, Cohn Fraction V; bovine crystalline serum albumin (Armour, batch CF/670); porcine and bovine globulins (Kekwick & Mackay, 1954; Fraction G1).

Measurements of pH. A Cambridge Instrument Co. pH meter was used.

The pH of the solvents systems (described below) and of the proteins in these systems was measured at room temperature without dilution with water. These readings, taken in the presence of high concentrations of organic solvents, afford only relative figures, the relation of which to pH measurements in aqueous solution is not well defined. This method allows, however, comparison of different solutions and permits observation of the gradual neutralization of acid by the addition of a base up to a desired end point. The response of the instrument to the pH changes under these conditions is rapid and definite. As the readings are taken at room temperature, no temperature correction is required. This method proved more convenient and as satisfactory as the usual practice of diluting a small volume of the solution with water and reading the pH in the diluted solution (Cohn *et al.* 1946).

Protein determination. The protein content of the preparation was determined by the micro-Kjeldahl method (Markham, 1942). Protein was calculated from the nitrogen figures by using the factor 6.25.

Fractionation of serum or plasma

(a) *With methanol.* The serum or plasma was mixed at room temperature with 10 vol. of methanol containing acid of the type and in the concentration found capable of retaining the albumin in solution. After $\frac{1}{2}$ hr. of vigorous stirring the mixture was centrifuged, the liquors were removed, and the precipitated globulins washed in the centrifuge with a volume of 90% methanol equal to half the original volume of serum or plasma. The liquor and washings were combined and, if not quite clear, clarified, as recommended by Cohn *et al.* (1946), by suspending some filter aid (e.g. diatomaceous earth) in the liquors and filtering through a filter, precoated with the filter-aid.

The clear solutions thus obtained were neutralized with NaOH, or with an organic base, e.g. diethylamine, to an apparent end point of pH 6, whereby a white flocculent precipitate of albumin was formed. An end point of pH 6 for neutralization is suitable when maximum yields are desired. An end point of pH 4.8–5 is, however, preferable when a colourless or only pale-yellow product is wanted. A slightly lower yield is usually obtained under the latter conditions, but some coloured impurities remain in the supernatants. The neutralizing agent was so chosen that it produced, in combination with the particular acid used,

a salt that is soluble in 90% methanol. The precipitated albumin was allowed to settle, was freed from the liquor by decantation and centrifuging, and was washed, while still in the centrifuge, successively twice with methanol and twice with ether, was dried in air and finally *in vacuo* over P_2O_5 at room temperature, and weighed. The albumin was thus obtained in the form of a free-flowing, non-hygroscopic powder.

The protein contents of the albumin preparations, calculated from Kjeldahl N, were in most cases 85–90%. The yields of albumin reported below were calculated from the actual protein contents.

(b) *With acetone.* This fractionation was performed generally as in (a) above, but with 2 vol. of acetone instead of 10 vol. of methanol. In this case only an organic base could be used in the neutralization of the acid–acetone liquors, as the sodium salts of the acids employed are insoluble in the aqueous acetone medium.

Tests for quality of albumin

(1) *Optical rotation.* Solutions (2%, w/v) of the specimens, in each case adjusted accurately to the desired pH, pH 4.8 and 7.4 by the addition of 0.1 N-HCl or 0.1 N-NaOH, respectively, were examined for their optical rotation, $[\alpha]_D^{20}$. Albumin that had been made by one of the conventional processes was compared with albumin that had been recovered from the acid–methanol process, and with albumin that had been denatured by treatment with 4 M-guanidine-HCl (20 hr.) or 8 M-urea (20 hr.).

(2) *Electrophoresis.* Zone electrophoresis was carried out on strips of filter paper (Whatman no. 1), with sodium barbitone buffer, pH 8.6. A 2% protein solution was applied to the paper. A constant-current power pack was used and a current of 0.8 ma/cm. width of paper was passed for 4 or 6 hr. At the end of the electrophoresis, the paper was dried at 105° for 5 min., and the protein stained with bromophenol blue (in 2% acetic acid). A continuous electrophoresis on paper according to Durrum (1951) was carried out on 1 g. of a specimen of albumin.

An electrophoresis in starch gel (borate, pH 8.6, according to Smithies, 1955) was carried out; two samples of whole pig serum and a sample of homologous albumin (made from the serum by using methanol-HCl) were run simultaneously in the same gel tray and stained by acid Schwartz 10B.

(3) *Solubility.* Small quantities of the specimen were mixed with water to give a 5% solution (2 ml.). The rate and completeness of dissolution were observed; to the solution, 0.1 N-NaOH was then added drop by drop until the pH was 7. During these additions the appearance of turbidity or precipitate when this occurred was taken as an indication of some degree of denaturation of the protein. Invariably, any such turbidity or precipitate redissolved at pH 7. This test, in which the solubility of albumin at different pH values is examined, is similar to one of the methods which Ehrenpreis, Maurer & Ram (1957) described as a criterion for the native state or degree of denaturation of albumin.

Immunological tests

Schwert (1957) and Kallee *et al.* (1957) have shown that albumin after recovery from a solution of ethanol-trichloroacetic acid still possesses the full antigenic properties

of native albumin, whereas denatured albumin has diminished or altered antigenic properties.

In this series of experiments the antigenic properties of bovine and porcine albumin were compared with homologous albumin that had been prepared by conventional processes. For this purpose, anti-albumin sera were obtained according to the method of Kabat & Mayer (1948). Porcine albumin (fraction V) and crystalline bovine albumin served as antigens. In each test two 2 kg. rabbits were immunized by three intraperitoneal injections, within 14 days, of 1 ml. of alum-precipitated albumin, corresponding to a total dose of 30 mg. of protein. Four weeks after the first injection, the rabbits were bled, serum was prepared from the blood, and stored at -20° , with merthiolate as preservative. The titre of the antiserum was determined in capillary precipitin tests against the homologous antigen, and the lowest concentration of a positive precipitin reaction was noted.

RESULTS

Solubility of proteins in acidified organic solvents

Aqueous solutions (1%) of the albumins or solutions in 0.9% sodium chloride of the globulins were mixed in appropriate proportions at room temperature with one of the solvents. When methanol or ethanol was used as solvent, the ratio of protein solution to solvent was 1:10 (by vol.); when acetone was used, it was 1:2. In the absence of acid, both the albumins and globulins are quantitatively precipitated. When a 1% albumin solution was mixed at room temperature, with stirring, with 10 vol. of methanol containing 0.07% of hydrochloric acid, a clear solution resulted. On adjusting this solution to pH 6, by the gradual addition of diethylamine dissolved in several volumes of methanol, a flocculent precipitate was formed, from which, after separating from the liquors, washing with methanol and ether, and drying, albumin was obtained as a fine white powder. The recovery in this case is nearly quantitative. Thus from 1.00 g. of porcine albumin (fraction V) 1.06 g. was recovered, from 1.00 g. of bovine albumin (fraction V) 0.95 g. was recovered, and from 0.50 g. of bovine crystalline albumin 0.475 g. was recovered. The type and approximate concentration of acid that enabled the albumin to be retained in solution were determined.

On mixing a 1% solution of globulin in 0.9% sodium chloride with acidified methanol (or ethanol or acetone), the protein is completely precipitated. This different behaviour of the two types of protein is unaltered when mixtures of albumin and globulin are treated with acidified solvents. Thus on mixing at room temperature a solution of 1.00 g. of porcine albumin and 1.00 g. of porcine globulin in 100 ml. of 0.9% sodium chloride with 1 l. of methanol containing 0.07% of hydrochloric acid, stirring the mixture for 30 min.

and separating the solution from the precipitate, the precipitate consisted of globulin, whereas albumin was recovered by neutralization of the solution. Typical yields from two such experiments were: (1) 0.90 g. of albumin and 0.85 g. of globulin recovered; (2) 0.80 g. of albumin and 1.00 g. of globulin recovered. This represents total recoveries of 87.5 and 90% respectively.

The acids in Table 1, in concentrations from 0.07 to 3%, retained albumin in solution in aqueous 90% methanol or ethanol, or in aqueous 66% acetone, but perchloric acid, picric acid, benzene- and toluene-*p*-sulphonic acid did not.

Preparation of albumin from porcine serum

Use of acidified methanol. Quantitative determinations of the albumin yield from serum, under the experimental conditions described above, served to explore the comparative power of different acids to retain albumin in solution, while precipitating the globulins. From the yields given in Table 1 (a) it is seen that hydrochloric acid and the strong organic acids retained high percentages of albumin in solution. The influence of acid concentration is illustrated for a strong acid, namely tartaric acid, and for a weak acid, acetic acid. With tartaric acid, the solubilizing effect increases from zero to quite high percentages with increasing concentration, whereas with acetic acid it is nil at low concentrations (0.2N), and even in high concentration (2N) the amount of albumin solubilized is small (30%). Sulphuric acid, although a strong acid, is not as effective as hydrochloric acid as a solubilizer.

Use of acidified acetone. Table 1 (b) shows the result of serum fractionation in acidified acetone. In the presence of the strongest acids (hydrochloric, formic, trichloroacetic acid), albumin is retained in solution; it is necessary, however, for this purpose to use a higher concentration of acid than is required in a methanol medium; even then the yields are not quite as high as with methanol. A higher acid concentration is necessary particularly with the hydroxy acids, e.g. lactic acid. With 1% lactic acid, 5% of albumin is retained in solution, and even with 10% lactic acid only 46% is retained.

Recovery of albumin from blood plasma. The fractionation of blood plasma was carried out in the same way as described for serum. A higher acid concentration (e.g. 0.14% of hydrochloric acid) was used, however, than in the serum fractionation (e.g. 0.07%) in order to compensate for the buffer action of the anticoagulants present. The yield of albumin was of the same order.

Fractionation of serum with added albumin. The fractionation of serum with acid-methanol allows under the most favourable conditions the recovery of a high percentage of the total albumin present.

The question then arises whether any given amount of albumin that may be present in serum or similar fluids can be isolated by this method. This was tested by comparing the recoveries of albumin from serum and from the same type of serum to which a known amount of albumin had been added. In a typical set of experiments, 30 ml. of porcine serum was fractionated with methanol and hydrochloric acid, as described above, and the albumin was isolated. Another portion of 30 ml. of the same serum was used to dissolve 0.50 g. of porcine albumin, and this solution was fractionated like the first one. In the first case, 0.72 g. of albumin was recovered, in the second 1.23 g. The increased recovery of albumin in the second case corresponded to the amount of added albumin. This result indicates that albumin can be separated from the globulins, independent of the albumin:globulin ratio.

Relation between the nature of the acids and their solubilizing effect. The solubilizing effect of the various acids used in the serum fractionation does not appear to be directly related to the degree of acidity that the solvent system shows in the presence of the different acids. Column 5 of Table 1 shows the pH readings of the acids in aqueous 90 %

methanol and 66 % acetone. When these figures are compared with the albumin yields, there is no strict correlation between pH and albumin yield. There may be a general trend in the sense that low pH means high yield, but the figures are not quite consistent in this respect. Notable exceptions are lactic acid, which is a good solubilizer at a relatively high pH, and sulphuric acid, which solubilizes albumin only to a small degree, in spite of the low pH of its methanolic solution. Other strong acids which have no solubilizing effect at all are benzene- and toluene-*p*-sulphonic acid, and perchloric acid. It must therefore be assumed that the nature of the anion is of decisive influence on the ability of an acid to create a medium that is a solvent for albumin. The number of acids that have been examined in this respect is too small to allow any definite conclusions to be drawn.

Properties of albumin prepared by acid-methanol fractionation of serum

Solubility. The albumin prepared by the methods described yields pale aqueous solutions of low viscosity; 10 % solutions are easily obtained. Five per cent solutions were subjected to the test of solubility at different pH, by the gradual addi-

Table 1. *Recovery of albumin from porcine blood serum*

(a) Serum (100 ml.) was fractionated with 1 l. of methanol containing acid of the type and concentration given below.

Acid	Concn. of acid (N)	Yield		pH of acidified 90% methanol†
		Albumin	(% of total present*)	
Hydrochloric	0.02	2.6	88	1.22
Trichloroacetic	0.05	2.6	88	1.58
Monochloroacetic	0.09	2.1	71	2.66
Formic	0.2	2.6	88	2.82
Lactic	0.11	2.4	81.5	3.10
Salicylic	0.2	2.4	81.5	2.57
Citric	0.36	1.9	64.5	2.81
Tartaric	0.1	0	0	3.08
Tartaric	0.2	0.5	17	2.96
Tartaric	0.4	2.0	68	2.90
Acetic	0.2	0	0	3.60
Acetic	2	0.8	27.5	2.80
Sulphuric	0.04	0.5	17	1.86

(b) Serum (100 ml.) was fractionated with 200 ml. of acetone containing acid of the type and concentration given below.

Acid	Concn. of acid (N)	Albumin	Yield (% of total present*)	pH of acidified 66% acetone†
Hydrochloric	0.04	2.0	68	1.41
Trichloroacetic	0.19	2.2	75	1.10
Formic	0.4	1.9	64	2.43
Formic	0.6	2.2	75	2.24
Lactic	0.11	0.14	4.5	3.07
Lactic	1.1	1.24	42	2.00

* Total albumin in porcine serum taken as 2.95 g./100 ml. (Cartwright, Smith, Brown & Wintrobe, 1948). Knill, Podleski & Childs (1958), from serum electrophoresis, gave the albumin content of serum as 3.4 g./100 ml. On the latter figure, the above percentage recoveries would be correspondingly smaller.

† Aqueous 90% methanol and 66% acetone, acidified with the type and concentration of acid, were as used in the serum fractionation.

tion of 0.1 N-sodium hydroxide. Solutions of those albumin specimens which had been made with methanol or ethanol as solvent and hydrochloric, formic or trichloroacetic acid, or with acetone in combination with trichloroacetic acid, did not show any opalescence or turbidity at any pH. On the other hand, solutions of albumin that had been prepared by using one of the other acids yielded turbidities or precipitates at or near pH 5. This was taken to indicate that such solvent systems denature the albumin to a certain degree.

It must be assumed that in the less successful solvent systems the albumin is denatured to such an extent that it is not completely soluble and that therefore the subsequent recovery of albumin is smaller.

On the strength of these findings, further tests were carried out only on albumin specimens that had passed the solubility test. Most of the following experiments were in fact carried out on albumin derived from fractionation with methanol and hydrochloric acid.

Electrophoresis. Albumin made by fractionation with methanol and hydrochloric acid showed, when tested by paper electrophoresis in 2% solution, the same rate of movement as albumin made from blood of the same animal by other established procedures. The freedom of the albumin from contamination with globulins was determined by continuous paper electrophoresis according to Durrum (1951). In an experiment with 1 g. of porcine albumin, this method failed to detect the presence of any globulin. As this method gives a strongly positive reaction in the presence of 0.5% of globulin, this indicates that considerably less than this percentage of globulin was present in the albumin tested. However, porcine albumin, made with methanol and hydrochloric acid, occasionally shows a small quantity of protein soluble in perchloric acid (0.7M overall final concentration), amounting to approx. 0.8% of the albumin. A

starch-gel electrophoresis (borate, pH 8.6, according to Smithies, 1955) was undertaken to show the nature of these proteins soluble in perchloric acid. The electrophoresis showed that these are from the slow α_2 which contains haptoglobin elements, known to be perchloric acid-soluble, and, moving ahead of the albumin, an acid glycoprotein. Such proteins were found only in porcine albumin and were encountered, not regularly, but only in occasional batches. The circumstances in which these proteins occur in porcine blood are not known.

The results obtained on electrophoresis indicate that, with the above-mentioned exception, albumin prepared by using acid-methanol has been freed from globulins to a high degree.

Optical rotation. The optical rotations of albumins of different origin and history were compared in solutions at pH 7.4 and 4.8 (Table 2). There was no difference in the rotation, either at pH 7.4 or 4.8, between crystalline bovine albumin, the same albumin after dissolution in methanol and hydrochloric acid, and reprecipitation, and albumin made by fractionation of bovine serum with methanol and hydrochloric acid. In the same way, the rotations were identical of porcine albumin, fraction V, and of albumin, made from porcine serum with methanol and hydrochloric acid. The rotations of any of these albumins after denaturation with urea or guanidine were found to be higher. As it is well known that the optical rotation of serum proteins rises on denaturation (Neurath & Bailey, 1953) these observations indicate that the methanol and hydrochloric acid process does not result in gross denaturation of the albumin.

Immunological tests. When anti-bovine albumin serum was tested in a capillary precipitin test against crystalline bovine albumin, a strongly positive reaction occurred at an antigen dilution of 1:500, decreasing to 1:50 000, being negative at 1:500 000. The strength of reaction and highest

Table 2. *Optical rotation of albumin specimens that had been subjected to different treatments*

Albumin			Optical rotation	
Animal species	Type	Treatment	pH	$[\alpha]_D^{20}$
Bovine	Crystalline	—	7.4	-59.4°
Bovine	Crystalline	Methanol-HCl-treated*	7.4	-59.2
Bovine	Crystalline	Methanol-HCl-made†	7.4	-59.2
Bovine	Crystalline	Denatured with guanidine	7.4	-65.0
Bovine	Crystalline	Denatured with urea	7.4	-65.5
Bovine	Crystalline	—	4.8	-63.7
Bovine	Crystalline	Methanol-HCl-made†	4.8	-64.0
Bovine	Crystalline	Urea-denatured	4.8	-72.0
Bovine	Crystalline	Guanidine-denatured	4.8	-74.2
Porcine	Fraction V	—	7.4	-58.3
Porcine	Fraction V	Methanol-HCl-made†	7.4	-58.6

* Cryst. albumin, dissolved in methanol and HCl and recovered by neutralization.

† Prepared by fractionation of serum with methanol and HCl.

dilution of positive reaction were identical for crystalline bovine albumin and the same albumin after methanol and hydrochloric acid treatment. In a test on anti-porcine albumin serum, strong reaction occurred at a porcine albumin dilution of 1:1000, and the highest antigen dilution giving positive reaction was 1:100 000. There was no difference between albumin (fraction V), the same albumin after methanol-hydrochloric acid treatment and albumin prepared by the methanol and hydrochloric acid method from serum. It may be concluded that the methanol-hydrochloric acid albumin acts as antigen towards an anti-albumin serum.

The above tests show that albumin made by the methanol and hydrochloric acid method is close to the native state and that it has been freed to a large extent from globulins. Albumin made by fractionation with methanol and formic acid appears to be in the native state, as judged by its solubility. The more stringent tests for denaturation have, however, not been applied to this type of product. Albumin made with alcohols acidified by various other acids, or with acidified acetone, appears in most cases to be partially denatured. The acid-alcohol method of serum separation is less successful with the globulins, as they appear to be denatured in the process.

Solubility of other proteins in acid-methanol

Of a number of other proteins studied, three other proteins, namely gelatin, globin and protamine, though insoluble in alcohols, are soluble in the acid solvent systems used in this work. On mixing a warm aqueous 5% solution of one of these proteins with 10 vol. of methanol, the protein is precipitated. If instead of methanol, methanol containing conc. hydrochloric acid is used (0.2% for gelatin, 1% for the other two), the solution remains clear and the protein can be recovered as described for albumin solution.

DISCUSSION

The successful isolation of albumin from blood serum with the aid of acidified polar solvents depends not only on the solubility of albumin in the solvent systems, but also on the preservation of this protein in its native state (as judged by solubility). Although it is well known that albumin, like other proteins, is prone to denaturation at room temperature in contact with organic solvents, the almost complete absence of damage to the protein in the present process appears to be due to the fact that the albumin, instead of being precipitated, is retained in solution as an ionized complex with the acid, i.e. in a state far removed from its isoelectric point.

Several authors, notably Schwert (1957), Korner & Debro (1956) and Kallee *et al.* (1957), have recently investigated the solubility of trichloroacetic acid precipitates of albumin in organic solvents, e.g. methanol, ethanol, acetone. Schwert has shown that the albumin is recovered in its native state from solutions in trichloroacetic acid-ethanol. He ascribed the solubilizing effect to 'the repression of ionization of carboxyl groups in the protein by the low pH of the trichloroacetic acid solution and the masking of cationic charges by ion-pair formation with trichloroacetate'. These authors' observations with trichloroacetic acid agree well with the present work and would appear to constitute only one case of the general principle outlined here. There is indeed no need to postulate for trichloroacetic acid some unique relationship between its marked ability to precipitate proteins in aqueous solution and its power to bring albumin into solution in ethanol. Another example of the general principle is to be found in the work of Peters (1958), who was able to extract albumin from its combination with its specific antibody globulin by the use of acidified ethanol.

Our observations show that albumin is not the only protein which is soluble in acidified alcohols, but that gelatin, globin and protamine, among other proteins, have this type of solubility. The well-known solubility of insulin and of corticotrophin in acidified alcohol, and that of β -lactoglobulin (Chibnall, Mangan & Rees, 1958), belong to this category. Study of further proteins may help to elicit the common factor responsible for this type of solubility and provide new means for protein identification. The possibility that proteins may be soluble in acidified solvents should not be overlooked in analytical procedures employing such media.

SUMMARY

1. The preparation of albumin from human or animal blood serum is described.
2. Serum or plasma is treated with acidified lower alcohols, whereby the globulins are precipitated, leaving the albumin in the supernatant liquid. It is shown that low concentrations of certain very strong acids are sufficient to retain substantially the whole of the albumin in solution. From this acid solution, by neutralization, the albumin is precipitated in a form almost undenatured and free from globulins. With weaker acids, or with acetone as solvent, the resulting albumin is in most cases partially denatured.
3. The conditions governing this separation are discussed and the results compared with recent similar work described in the literature.
4. Some observations on the solubility of other proteins in acidified alcohols are mentioned.

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The Endogenous Citric Acid-Cycle Intermediates and Amino Acids of Mitochondria

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It is well known that mitochondria isolated from a variety of animal tissues absorb oxygen when incubated without added substrate (e.g. Minnaert, 1960). Since a large proportion of the enzymes of the citric acid cycle are found in the mitochondrial fraction of the cell (Hogeboom, Claude & Hotchkiss, 1946; Schneider & Hogeboom, 1950; Chappell & Perry, 1953; Sacktor, 1955), it is probable that the endogenous substrates of mitochondria, if not citric acid-cycle intermediates, are capable of conversion into these compounds. Indeed, citrate has already been identified as an endogenous substrate of rat-liver mitochondria (Schneider, Striebig & Hogeboom, 1956). Other work (Minnaert, 1960; Weinbach, 1961) has shown that the endogenous respiration of rat-liver mitochondria is associated with the esterification of inorganic phosphate and may be inhibited by malonate and arsenite. Thus the available evidence supports the view that endogenous respiration involves substrate oxidation by way of the citric acid cycle.

This paper describes the extraction and measurement of citric acid-cycle intermediates and amino

acids in well-washed mitochondria obtained from a variety of animal tissues. It was found that all types of mitochondria contained glutamate and aspartate, whereas citric acid-cycle intermediates had a more limited distribution.

METHODS

Preparation of mitochondria. For details of the histological methods used to define the mitochondrial fraction of the cell see Bellamy (1958).

Rat and mouse liver. Mitochondria were isolated from rat and mouse liver by the method of Werkheiser & Bartley (1957), except that the dissolved CO₂ was not removed from the sucrose solution.

Rat and mouse skeletal muscle. The animals were killed by a blow on the head. The hind legs were skinned, severed at the pelvis and placed in ground ice. About 15 min. later muscle removed from the upper leg was passed through a chilled mincer. The mince was suspended in 10 vol. of 0.25 M-sucrose solution and homogenized (pestle moved up and down 10 times). The homogenate was centrifuged at 1000g for 10 min., the supernatant fluid decanted, stored in ice and the residue suspended in the original volume of 0.25 M-sucrose solution. The first centrifuging was then repeated. The combined supernatant fluids were centrifuged at 1000g for 10 min. The residue from this pro-

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