The Estimation of Acetone Bodies

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In a study of the pathogenesis of bovine ketosis it was felt desirable to be able to follow in some detail the changes in all three ketone bodies (acetone, acetoacetic acid and β-hydroxybutyric acid) which have been shown by various workers to occur in excessive quantities in blood, milk and urine during the course of that disorder. Hitherto, little or no attempt has been made at complete differentiation as both acetone and acetoacetic acid are usually determined in the one fraction. Moreover, *iso*-propanol which we have found (Robertson, Thin & Stirling, 1950) to be present in this disease and in pregnancy toxaemia of ewes, has been unsuspected hitherto in such conditions and, as it is oxidized to acetone by chromic acid, its presence will undoubtedly have influenced the estimation of total ketones by various methods. In this paper the term 'Total Acetone Bodies' is used, therefore, to include acetone, acetoacetic acid, β-hydroxybutyric acid and *iso*-propanol.

The accurate determination of free acetone in the presence of acetoacetic acid is difficult. Van Slyke's method, using Denigès reagent (Van Slyke, 1917, 1929) which has frequently been utilized in ketosis studies, cannot be used to estimate free acetone; in addition, it is not very sensitive as we have found that no precipitate is formed with small amounts of acetone bodies of the order of 5 mg./100 ml. or less. Moreover, using this method, *iso*propanol appears to be oxidized to acetone partly in the acetone plus acetoacetic acid fraction, and partly in the β-hydroxybutyric acid fraction, so making the accurate determination of the individual acetone bodies impossible.

Attempts at using the distillation methods of Shaffer & Marriott (1915) and Behr (1928, 1940) for free acetone by distillation under reduced pressure, or by blowing a fine stream of air through the solution at room temperature, in order to prevent acetoacetic acid decomposing, gave poor results, as with quantities likely to be present in biological fluids, only a small proportion of the acetone could be recovered. The method of Werch (1940, 1941), utilizing diffusion into Nessler's solution in Conway microdiffusion units, though providing a very delicate qualitative test, did not give accurate quantitative results; the time for the appearance of the precipitates varied and they could not be estimated gravimetrically with any degree of success. The following method was therefore devised, combining with some modification the oxidation technique of Greenberg & Lester (1944), the diffusion method of Werch (1940, 1941) and Seifert (1948), and the colorimetric technique of Behr & Benedict (1926). It depends on the development of an orange to red coloration when an alkaline solution of salicylic aldehyde is left in the presence of acetone, the depth of colour formed being directly proportional to the amount of acetone present. This can be used to estimate free acetone, the acetone formed by hydrolysis of acetoacetic acid, and that formed by chromic acid oxidation of β-hydroxybutyric acid and of *iso*propanol.

METHODS AND RESULTS

Apparatus. Microrefluxing apparatus (Greenberg & Lester, 1944); Conway microdiffusion units.

Reagents. Approx. 0·15 N-$Ba(OH)_2$ and approx. 2·5% (w/v) $ZnSO_4.7H_2O$ (Greenberg & Lester, 1944); 20 N-H_2SO_4; 10% (w/v) $K_2Cr_2O_7$; 1·5% (w/v) $K_2Cr_2O_7$ in 15·6 N-H_2SO_4; 20% (w/v) acetic acid; 4 N-KOH; salicylic aldehyde supplied by British Drug Houses Ltd.

Colour reagent. To 1 ml. of salicylic aldehyde are added 8 ml. of 4 N-KOH and the solution well mixed; 2 ml. are used for each estimation.

Preparation of standard graphs. A standard solution of acetone (3 ml.), prepared according to Behr & Benedict (1926) and containing 0–10 mg. acetone/100 ml. of solution are placed in the outer chamber of a Conway dish with a few drops of 20% (w/v) acetic acid; 2 ml. of the colour reagent are placed in the inner chamber. The lid is put on firmly, after greasing the rim, and the dish is left 0·5–3 hr. in an incubator at 37°, or any other standard time and temperature that is convenient; alternatively, it may be kept at room temperature overnight, when the maximum colour is obtained regardless of temperature. It should not be left standing, however, more than 20 hr. as the colour reagent tends to blacken after this interval. A blank is run using water instead of the standard solution.

After the requisite time 0·5 ml. of the coloured solution in the central chamber is added to 2 ml. of water in a test tube, and the resulting solution read in the Spekker photoelectric colorimeter against the blank similarly prepared, using 2 ml. cells and a blue-green filter (Ilford 603).

Fig. 1 shows a graph prepared by estimating known amounts of acetone. It will be seen that Beer's Law is strictly adhered to over the range 0–10 mg./100 ml. Table 1 shows that results with standard acetone solutions are reproducible within ±4%.

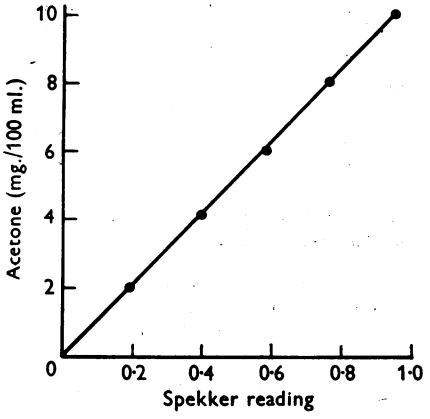

Fig. 1. Standard curve for acetone, measuring the colour produced with alkaline salicylic aldehyde against a blank after standing 2 hr. at 37°. Filter: blue-green, Ilford 603.

Table 1. *Reproducibility using standard solutions of acetone*

Time interval (hr.)	Theoretical result (mg./100 ml.)	Acetone found (mg./100 ml.)	Error (%)
2	7·92	7·80	−1·5
2	7·92	8·00	+1·0
Overnight	7·92	7·70	−2·8
Overnight	7·92	8·10	+2·3
2	1·98	1·90	−4·0
2	1·98	2·03	+2·5
Overnight	1·98	2·00	+1·0
Overnight	1·98	2·05	+3·5

APPLICATION TO BIOLOGICAL MATERIAL

Blood

Protein precipitation: to 2 ml. of distilled water in a centrifuge tube 1 ml. of blood is added and the solution well mixed; 3 ml. of 0·15 N-$Ba(OH)_2$ are then added, followed by 3 ml. of 2·5% (w/v) $ZnSO_4.7H_2O$. The solution is well mixed, the tube stoppered and centrifuged. The clear supernatant liquid is used for estimating acetone, acetone plus acetoacetic acid and 'Total Acetone Bodies'.

Free acetone. A 3 ml. sample of the supernatant fluid is placed in the outer chamber of a greased Conway dish and the estimation carried out as above. Owing to dilution during precipitation and oxidation, it is necessary to multiply the results obtained from the standard graphs by the factor 9 to get the results in mg. acetone/100 ml. of original solution.

Free acetone can also be estimated without precipitation of protein if so desired. Recovery experiments which were carried out using the complete technique of precipitation etc., on standard solutions in water and blood showed the same limits of accuracy as in direct estimation of standard solutions (Table 2).

Table 2. *Recovery of added acetone*

(The blank was 0 mg./100 ml. in each case.)

Acetone added (mg./100 ml.)	Acetone found (mg./100 ml.)	Error (%)
Standard acetone solution		
7·92	7·80	−1·5
7·92	8·00	+1·0
7·92	8·10	+2·3
Standard solutions in blood		
7·92	8·01	+1·1
7·92	7·83	−1·1
7·92	8·10	+2·3
15·84	16·20	+2·3
Standard solutions in blood (direct method)		
7·92	8·00	+1·0
7·92	8·10	+2·3
7·92	8·20	+3·5
15·84	15·50	−2·1

Acetoacetic acid. Acetoacetic acid is determined by difference, the free acetone determined as above being subtracted from the acetone plus acetoacetic acid value, obtained as follows.

Acetone plus acetoacetic acid. A 3 ml. sample of the supernatant solution is placed in the microrefluxing apparatus and 1·1 ml. of 20 N-H_2SO_4 and a glass bead are added. The apparatus is shut firmly after applying a very thin film of grease to the ground-glass joint, the flow of water is started and the contents of the reflux tube boiled for 5 min. The apparatus is then cooled rapidly under running water, tipped to mix well, and 3 ml. of the solution are transferred to the outer chamber of a Conway dish for the determination of the acetone present. The factor in this case is 12·3.

Recovery experiments were carried out as before, using commercial grade ethyl acetoacetate purified by distillation under reduced pressure. The results obtained (Table 3) came within ±4% of the theoretical amounts.

Table 3. *Recovery of acetoacetic acid, expressed as acetone*

Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Error (%)
Standard solutions			
5·09	5·04	0	−0·9
5·09	5·04	0	−0·9
10·18	10·10	0	−0·8
10·18	9·84	0	−3·3
19·57	19·68	0	+0·6
19·57	19·68	0	+0·6
Standard solutions in blood			
5·09	5·16	0	−1·4
5·09	5·04	0	−0·9
5·76	18·45	12·92	−4·0
5·76	18·45	12·92	−4·0
11·52	24·60	12·92	+1·4
11·52	24·60	12·92	+1·4

Table 4. *Recovery of* iso*propanol, expressed as acetone*

Amount added (mg./100 ml.)	Microdistillation: Amount found (mg./100 ml.)	Microdistillation: Blank (mg./100 ml.)	Microdistillation: Error (%)	Method of Friedmann (1938): Amount found (mg./100 ml.)	Method of Friedmann (1938): Blank (mg./100 ml.)	Method of Friedmann (1938): Error (%)
			Standard solution			
7·43	7·38	0	−0·6	7·25	0	−2·4
7·43	7·38	0	−0·6	7·50	0	+0·9
7·43	7·38	0	−0·6	7·25	0	−2·4
			Standard solution in blood			
7·43	11·81	4·31	+0·9	7·5	0	+0·9
7·43	11·81	4·31	+0·9	7·25	0	−2·4
7·43	11·44	4·31	−4·0	7·25	0	−2·4

iso*Propanol.* *iso*Propanol is oxidized to acetone under the conditions normally employed for estimating 'Total Acetone Bodies' and so is included in this term. It can also be estimated separately using a modification of Friedmann's (1938) method in which the *iso*propanol alone is oxidized to acetone. A Markham steam distillation apparatus is used in this procedure, except in the case of blood, as it is more convenient for small quantities, as well as being quicker and easier to clean between samples.

A 50 ml. sample of blood is transferred to a large conical flask with 100 ml. of distilled water, 10 ml. of $HgSO_4$ solution (Friedmann, 1938) are added and the solution mixed, then 15 ml. of 10% (w/v) sodium tungstate and a little wax to prevent frothing. The solution is well shaken and steam-distilled, about 100–200 ml. of distillate being collected.

The distillate is washed into a round-bottomed flask, 5 ml. of $HgSO_4$ solution added and excess 10% (w/v) $Ca(OH)_2$ suspension till the solution is alkaline. This solution is distilled directly into a conical flask, about 100 ml. of distillate being collected. 10 ml. of 20 N-H_2SO_4 and excess $K_2Cr_2O_7$ are added to the distillate and the flask, loosely corked, is either placed on a drying oven (about 50°) for 2 hr., or left overnight at room temperature.

The solution is then washed into a round-bottomed flask and about 50 g. $MgSO_4$ added. The solution is distilled direct, a little less than 100 ml. distillate being collected; this is made up to 100 ml. with distilled water in a graduated flask and the acetone content determined by placing 3 ml. of the resulting solution in the outer chamber of a Conway dish and proceeding as above, using the factor 5 for the calculation.

Recovery experiments are shown in Table 4. The microrefluxing method used gave results similar to those obtained with Friedmann's method.

β-Hydroxybutyric acid. β-Hydroxybutyric acid is determined by difference, the acetone plus acetoacetic acid plus *iso*propanol values being subtracted from the value for 'Total Acetone Bodies', obtained as follows: a 3 ml. sample of the supernatant solution is placed in the microrefluxing apparatus with 0·7 ml. of 1·5% (w/v) $K_2Cr_2O_7$ in 15·6 N-H_2SO_4, and a glass bead. The apparatus is closed firmly as before, the water flow started, and the contents boiled for 10 min. The solution is taken off the boil and 0·4 ml. of 10% (w/v) $K_2Cr_2O_7$ run down the cold finger into the solution by means of a syringe. The apparatus is closed firmly again and the solution boiled for a further 10 min., cooled, tipped to mix the contents and 3 ml. transferred to the outer chamber of a Conway dish and the acetone content determined. The factor here is 12·3.

The accuracy of the method for 'Total Acetone Bodies' when done on acetone and acetoacetic acid solutions was within ± 3% (Table 5). Estimations

Table 5. *Recovery of 'Total Acetone Bodies'*

Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Error (%)
	Acetone in standard solutions		
9·50	9·64	0	+1·5
9·50	9·32	0	−1·9
9·50	9·32	0	−1·9
23·76	23·37	0	−1·6
23·76	23·37	0	−1·6
23·76	23·99	0	+0·9
	Acetone in blood		
9·50	12·97	3·20	+2·8
9·50	12·42	3·20	−2·9
9·50	12·67	2·95	+2·3
23·76	27·06	3·20	+0·4
23·76	27·68	3·20	+3·0
23·76	26·45	2·58	+0·4
	Acetoacetic acid in standard solutions		
5·76	5·78	0	+0·3
11·52	11·56	0	+0·3
	Acetoacetic acid in blood		
5·76	27·48	21·89	−3·0
11·52	33·33	21·89	−0·7

of β-hydroxybutyric acid in the quality obtainable, namely, British Drug Houses Laboratory Reagent, gave a practically consistent error of approx. 40% in both standard solutions and in blood (Table 6). Various modifications of this method which were tried, such as altering the concentration of chromic acid, varying the time of oxidation and varying the concentration of β-hydroxybutyric acid, all failed to give any increase in the percentage recovery. It was thought that part of this discrepancy might be due to impurity, but at our request Messrs British Drug Houses Ltd. kindly examined their product which we had used, the sodium salt of β-hydroxybutyric

acid, and reported that it gave the expected 78 % yield of acetone when examined by the method of Greenberg & Lester (1944) and that an approximate determination of purity by precipitation of the sodium with hydrochloric acid in ethanol, followed by the removal of the acid into ether, gave a result of about 98 %. A determination of the sodium content from sulphated ash also gave results equivalent to 98 % purity.

Table 6. *Recovery of β-hydroxybutyric acid, expressed as acetone*

Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Error (%)
	Standard solutions		
5·08	3·07	0	39·6
5·08	3·07	0	39·6
5·08	3·08	0	39·4
10·15	6·13	0	39·6
10·15	6·13	0	39·6
10·15	6·26	0	38·3
15·24	9·10	0	40·3
15·24	9·10	0	40·3
15·24	9·35	0	38·6
	Standard solutions in blood		
5·08	7·38	4·31	39·6
5·08	5·41	2·34	39·6
5·08	5·29	2·34	41·9
10·15	9·84	3·73	39·8
10·15	9·84	3·73	39·8
10·15	10·08	3·73	38·3
15·24	13·16	3·73	38·1
15·24	12·79	3·73	40·5
15·24	13·16	3·73	38·1

From our results it would appear, therefore, that chromic acid only oxidizes 60 % of the β-hydroxybutyric acid present to acetone under the above experimental conditions, as contrasted with the 78 % yield of acetone obtained by Greenberg & Lester (1944) using rather different conditions. As our method of determination yields results consistently 40 % low, then to determine the true amount of β-hydroxybutyric acid in a sample the value obtained by subtraction must be multiplied by 5/3. This corrected value added to the previously determined acetone plus acetoacetic acid plus *iso*propanol value will then give the true amount of 'Total Acetone Bodies' present.

Rumen contents

The rumen contents are strained through surgical gauze to remove large pieces of food, and the moderately clear liquid is used for the estimations. The protein is precipitated as for blood and the procedure and factors are the same except for *iso*propanol, where the factor is 5 and the procedure as follows: a 10 ml. sample of the filtered rumen liquor is placed in a Markham steam-distillation apparatus with 2 ml. of $HgSO_4$ solution (Friedmann, 1938) and 2 ml. 10 % (w/v) sodium tungstate solution, and steam distilled, about 100 ml. of distillate being collected. This is repeated with a further 10 ml. of liquid and the distillates added.

The combined distillates are washed into a round-bottomed flask and 5 ml. of $HgSO_4$ solution and excess 10 % (w/v) $Ca(OH)_2$ suspension added. This solution is distilled direct and the procedure then follows that given for blood.

Milk

As milk contains many reducing substances it is necessary to dilute the solution still further in order to get full oxidation of β-hydroxybutyric acid and *iso*propanol to acetone. This is achieved by precipitating the protein as follows: a 0·4 ml. sample of milk is added to 4·6 ml. of distilled water, 2 ml. of 0·15 N-$Ba(OH)_2$ solution and 2 ml. of 2·5 % (w/v) $ZnSO_4.7H_2O$ are added. The tube is stoppered after mixing the contents and centrifuged as before.

The technique employed is the same as for blood except in the case of *iso*propanol where the method is similar to that used for rumen contents.

The factors are, for free acetone 22·5, for acetone plus acetoacetic acid and 'Total Acetone Bodies' 30·75 and for *iso*propanol 5.

Urine

The procedure and factors are the same as for blood except in the case of *iso*propanol, where the rumen technique is used, and the factor is 5.

In certain acute cases of acetonaemia, the content of 'Total Acetone Bodies' in urine rises to several hundred mg./100 ml. In these cases it is advisable to dilute the protein-free liquid still further before oxidation.

Recovery experiments carried out on milk and urine using added acetone gave the results shown in Table 7.

Table 7. *Recovery of added acetone from milk and urine*

(The blank value was 0 mg./100 ml. in each case.)

Acetone added (mg./100 ml.)	Acetone found (mg./100 ml.)	Error (%)
	Milk	
7·92	7·88	−0·5
7·92	7·88	−0·5
7·92	7·88	−0·5
15·84	16·20	+2·3
15·84	16·20	+2·3
	Urine	
7·92	7·83	−1·1
7·92	7·83	−1·1
7·92	8·10	+2·3
15·84	15·93	+0·5
15·84	15·75	−0·5

Interference

The following substances were tested for interference in the estimation of acetone bodies: acetic acid, lactic acid, sodium chloride, cholesterol, urea, formaldehyde and acetaldehyde. With the exception of acetaldehyde, the results obtained showed there was no interference. This compound in concentrations as low as 3 mg./100 ml. reacts with the colour

reagent to give a slightly opaque orange solution. The interference is lessened a little after boiling with chromic acid, but not sufficiently to allow of an accurate determination even of total ketones in the presence of such amounts of this material.

Recovery from mixed solutions

From the preceding tables it can be seen that using standard solutions of one of the 'Acetone Bodies', the errors lay within the range ± 5 % except in the case of β-hydroxybutyric acid where the percentage yield of acetone was only 60, a discrepancy which is overcome by the use of the appropriate factor.

Experiments were carried out with similar small errors on standard mixtures of 'Acetone Bodies' after complete precipitation and oxidation (Table 8).

Estimations were also carried out on standard mixtures in blood and in rumen contents (Table 9), and the experimental errors were again found to lie within the same range. However, the concurrent experiments carried out using Denigès' method gave percentage errors with a much wider range.

DISCUSSION

From the results it can be seen that the method is readily applicable to two significant biological materials, namely, blood and rumen contents, in acetonaemia and pregnancy toxaemia. It can equally well be used for milk and urine with no appreciable change in precipitation and oxidation technique.

Although by no means perfect it is the first relatively simple method of obtaining a fairly adequate and reasonably accurate differentiation of the various ketone bodies involved in ruminant

Table 8. *Recovery of mixtures of ketone bodies using standard solutions*

Exp. no.	Mixture	Theoretical result (mg./100 ml.)	Amount found (mg./100 ml.)	Error (%)
1	Acetone	7·92	7·75	−2·1
	Acetoacetic acid	3·81	3·68	−3·4
	*iso*Propanol	7·43	7·50	+0·9
	β-Hydroxybutyric acid	5·08	5·30	+4·3
2	Acetone	7·92	7·75	−2·1
	Acetoacetic acid	3·81	3·81	0
	*iso*Propanol	7·43	7·15	−3·7
	β-Hydroxybutyric acid	10·15	9·99	−1·6
3	Acetone	15·84	15·30	−3·4
	Acetoacetic acid	7·62	7·45	−2·2
	*iso*Propanol	14·86	14·00	−5·7
	β-Hydroxybutyric acid	15·45	14·60	−5·5

Table 9. *Recovery of mixtures*

Exp. no.	Mixture	Amount added (mg./100 ml.)	Amount found (mg./100 ml.)	Blank (mg./100 ml.)	Amount recovered (mg./100 ml.)	Error (%)
			In blood			
1	Acetone	7·92	7·87	0	7·87	−0·6
	Acetoacetic acid	3·81	3·63	0	3·63	−5·8
	*iso*Propanol	7·43	7·25	0	7·25	−2·4
	β-Hydroxybutyric acid	5·08	9·60	4·30	5·30	+4·3
2	Acetone	7·92	8·03	0	8·03	+1·0
	Acetoacetic acid	3·81	3·53	0	3·53	−7·0
	*iso*Propanol	7·43	7·25	0	7·25	−2·4
	β-Hydroxybutyric acid	10·15	18·90	8·20	10·70	+5·4
3	Acetone	15·84	15·30	0	15·30	−3·4
	Acetoacetic acid	7·62	7·46	0	7·46	−0·2
	*iso*Propanol	14·86	15·00	0	15·00	+0·9
	β-Hydroxybutyric acid	15·45	23·17	7·18	15·99	+3·5
			In rumen contents			
4	Acetone	7·92	7·87	0	7·87	−0·6
	Acetoacetic acid	3·81	3·93	0	3·93	+3·2
	*iso*Propanol	7·43	7·25	0	7·25	−2·4
	β-Hydroxybutyric acid	5·08	8·08	3·17	4·91	−3·3
5	Acetone	15·84	15·50	0	15·50	−2·1
	Acetoacetic acid	7·62	7·10	0	7·10	−6·8
	*iso*Propanol	14·86	14·40	0	14·40	−3·1
	β-Hydroxybutyric acid	15·45	18·50	3·03	15·47	+0·1

pathology and should prove of value in throwing further light on the pathogenesis of various conditions in which acetonaemia is a prominent feature.

SUMMARY

1. A method has been devised for the estimation as acetone of the individual ketone bodies—acetone, acetoacetic acid, β-hydroxybutyric acid and *iso*propanol—within the range 0–120 mg. acetone/100 ml.

2. The basis of the method is the diffusion of acetone into an alkaline solution of salicylic aldehyde with the production of an orange-red colour, the intensity of which is measured in a photoelectric colorimeter.

3. The application of the method to biological materials such as blood, milk, urine and rumen liquor is described.

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The Preparation and Properties of β-Glucuronidase

3. FRACTIONATION AND ACTIVITY OF HOMOGENATES IN ISOTONIC MEDIA

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The first paper in this series (Kerr & Levvy, 1951) dealt with the fractionation of glucuronidase activity in water homogenates of mouse liver and other organs which is brought about by buffering to slightly acid pH. Changes in the glucuronidase activity of a tissue with the state of proliferation (Levvy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949, 1950) were seen to be confined to the non-precipitable fraction. In the second paper (Walker & Levvy, 1951), it was shown that an identical fractionation of mouse-liver glucuronidase in untreated water homogenates could be achieved on the high-speed centrifuge, and that the sedimentable fraction was associated with cytoplasmic granules of all sizes. The use of water in preparing tissue homogenates caused pronounced osmotic swelling of the nuclei and other subcellular particles, leading sometimes to disruption. Homogenizing in isotonic media tends to preserve these bodies, and the present paper deals with the partition of glucuronidase activity between sedimentable material and the suspension fluid after homogenizing mouse liver in isotonic sodium chloride or sucrose solution. It was found that liver preparations did not display their full glucuronidase activity until a considerable proportion of the enzyme had been brought into solution.

METHODS

Preparation and fractionation of the enzyme. Unless stated to the contrary, the mouse liver was ground for 1 min. in the glass homogenizer (average speed 5000 rev./min.) with sufficient water, 0·17 M-NaCl or 0·25 M-sucrose solution to give a final tissue concentration of 10%, and samples were taken for assay. Following the procedure already described (Walker & Levvy, 1951) fractionation was then carried out either by centrifuging at a high speed (25000 *g* for 15 min.), or by adjusting the pH to 5·2 with acetate or citrate buffer (final concentration 0·1 N) and centrifuging at a low speed (1500 *g* for 15 min.). In the majority of experiments, the figure for sedimented enzyme activity was found by difference from the figures for total and unsedimented enzyme, the assumption being made that the latter was uniformly distributed between supernatant and precipitate. Control experiments to test this assumption, similar to those carried out with water homogenates (Walker & Levvy, 1951), showed that it also held for the homogenates in isotonic media.

Enzyme assay. After homogenizing liver in isotonic media, the enzyme associated with the subcellular particles did not display its full potential activity (see below). Unless otherwise stated, therefore, such homogenates were treated prior to assay by shaking with Ballotini, grade 12 (Chance Bros. Ltd.) in the Mickle tissue disintegrator (Mickle, 1948) for 10 min. When the sedimented material from these homogenates was subjected to direct assay, it was found sufficient to rehomogenize in water.