were 79.4, 80.9, 77.6 and 81.2 respectively. The mixture of proteins in all diets had, therefore, a high biological value which must have been due to supplementation amongst the proteins. Not much more than 40% of the total proteins in the diets were of animal origin.

3. The true digestibility coefficients of the four diets were 84.5, 86.3, 83.5 and 84.6 respectively.

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Studies on the Acetone-Butanol Fermentation

4. ACETOACETIC ACID DECARBOXYLASE OF *CL. ACETOBUTYLICUM* (BY)

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Although the enzyme systems responsible for the fermentation of pyruvic acid and of glucose by *Cl. acetobutylicum* are so unstable as a whole that, in the absence of the substrate, their activity cannot be preserved in cell suspensions for more than a few hours [Davies, 1942], one enzyme has been found to be extremely stable; this is acetoacetic acid decarboxylase, the enzyme responsible for the decarboxylation of acetoacetic acid to acetone. The quantitative nature of the reaction, its optimum pH, and the effect of the age of the cells on their decarboxylase activity, have already been described; also the insensitivity of the enzyme to O₂, its ability to withstand acetone, and the fact that it can be extracted cell-free from the acetone powder [Davies, 1942]. In this paper the purification and properties of the isolated enzyme will be described. The parallel isolation of the lysine decarboxylase of *Escherichia coli* will be published later [Gale & Epps, 1943].

METHODS

Preparation of acetoacetic acid. 13 g. of redistilled ethyl acetoacetate were dissolved in 100 ml. N NaOH and kept at room temperature for 40 hr.

The ice-cooled solution was acidified with H₂SO₄ (25 ml. of 20% by vol.); the acetoacetic acid was twice extracted with 150 ml. ether and the ether evaporated at room temperature. From the residue neutralized with N NaOH the unchanged ester was removed by extraction with ether; finally the ether was evaporated *in vacuo*. After standardization by measuring the CO₂ evolved when a portion was incubated with acetone-treated cells of *Cl. acetobutylicum* in Warburg manometers, the concentration was adjusted to M/2.

Preparation of acetone-treated cells. 5 l. flasks containing 4½ l. of 2% glucose-liver medium [Davies & Stephenson, 1941] were sown with 80 ml. of a vigorously fermenting culture of *Cl. acetobutylicum* (BY) on 5% maize-meal mash, and were then incubated at 37° until the Rothera acetone test was intense (about 40 hr.). The culture was then strained through muslin and the cells collected in a Sharples super-centrifuge. They were washed once on the ordinary centrifuge with about 400 ml. distilled water and suspended in 50–100 ml. distilled water. This suspension was blown rapidly from a pipette into 10 vol. of vigorously stirred acetone; after a few minutes the precipitate was collected on a

Buchner funnel, washed with acetone and with ether, and sucked dry.

Conditions for the determination of enzyme activity

The optimum substrate concentration, and the optimum pH for decarboxylation, were determined for both fresh and acetone-treated cells. Q_{CO_2} for the decarboxylation was measured by Warburg manometers.

Effect of substrate concentration. It is seen (Fig. 1) that the affinity of the enzyme for its substrate is fairly low, and is slightly lower for fresh cells than

Fig. 1. Effect of substrate concentration on the rate of decarboxylation by fresh and acetone-treated cells. Warburg manometers contained 0.3 ml. 2M acetate buffer pH 4.5 (for fresh cells) or pH 5.0 (for acetone-treated cells), sufficient M/2 acetoacetic acid to give concentrations of M/15 to M/1000, and in the side bulb 0.1 ml. cell suspension; total vol. 3.0 ml.; temp. 37.5°; gas phase air.

for acetone-treated cells, which require M/20 acetoacetic acid to saturate the enzyme, the half-speed concentration being 0.007M.

Effect of pH. This is shown in Fig. 2; curves 1 and 2 were obtained with two different suspensions of fresh cells and the optimum pH differs considerably for the two. The optimum pH for certain amino-acid decarboxylases of other micro-organisms also varies similarly [Gale, personal communication]. The optimum pH for the decarboxylation by acetone-treated cells is seen to be 5.0.

Estimation of the activity of the enzyme preparations. Conditions of optimum activity were used, the pH being 5.0 and the final acetoacetic acid concentration $\leq$ M/20. Decarboxylation was measured in Warburg manometers containing in a total volume of 3.0 ml., 0.3 ml. 2M acetate buffer of pH 5.0 and 0.5 ml. M/2 acetoacetic acid; the gas space contained air and the temperature was 37.5°.

The rate of CO_2 evolution has been correlated with the C content of the enzyme preparations, determined by the method of Van Slyke & Folch [1940], and the activities are expressed as μ l. CO_2 /hr. $\times$ mg. C, called $Q_{CO_2}^C$. One enzyme unit (E.U.) has

Fig. 2. Effect of pH on the rate of decarboxylation by fresh and acetone-treated cells. Warburg manometers contained 0.3 ml. 2M acetate buffer of the desired pH, 0.5 ml. M/2 Na acetoacetate, and in the side bulb 0.1 ml. cell suspension (for fresh cells 4.4 mg. dry wt. cells/ml.; for acetone-treated cells 5 mg. acetone powder/ml.); total vol. 3.0 ml.; temp. 37.5°; gas phase air.

been defined as the amount which will liberate 100 μ l. CO_2 in 5 min. at 37.5° under the optimum conditions.

Extraction of the enzyme

Distilled water at 37° was the most satisfactory extractant. The pH was not critical but at pH 4.0 extraction became very poor (Table 1). Prolonged

Table 1. *Extraction of the enzyme*

5 mg. of acetone-treated cells were incubated for 4 hr. at 37° with 5 ml. of the following: (a) distilled water, (b) 0.9% NaCl, (c) M/100 acetate pH 4.0, (d) M/100 acetate pH 5.0, (e) M/100 acetate pH 6.0, (f) M/75 phosphate pH 7.0, (g) M/100 borate pH 8.0, (h) M/100 borate pH 9.0. The suspensions were then centrifuged and a suitable portion of the supernatant tested for decarboxylase activity.

Extracting agent	Buffer at pH								
	Water	0.9% NaCl	4.0	5.0	6.0	7.0	8.0	9.0	
% initial activity extracted in 4 hr. at 37°	52	41	6	51	50	38	29	25	

incubation results in bacterial contamination of the acetone powder suspension, hence a few drops of capryl alcohol were added to one half the suspension to check this; it will be seen that whereas in

24 hr. 95% of the enzyme was extracted in the presence of capryl alcohol, 140% was extracted in its absence (Fig. 3). This extra enzyme could hardly

Fig. 3. Rate of extraction of enzyme from acetone powder at 37°. 25 mg. acetone-treated cells were suspended in 5 ml. water and incubated at 37°; to a similar suspension was added one drop of capryl alcohol, with shaking, and this also was incubated at 37°. Samples were withdrawn at intervals for the determination of decarboxylase activity in the extract.

have resulted from bacterial growth; and it is probable that it was present all the time but that its activity was somehow masked. Fig. 4 shows the variation of the total and of the extractable activity with the temperature of incubation. Heating the suspension to 70° for 30 min. almost doubled the

Fig. 4. Variation of total and extractable activity with temperature of incubation. Suspensions containing 60 mg. acetone powder in 2 ml. water were incubated at various temperatures for 30 min. and the decarboxylase activity of the extract was then determined.

total activity of the suspension, although at no temperature is more than about half the total activity extractable. This increase in activity cannot

be due to production of new enzyme but must be due to freeing of enzyme previously masked. Probably the enzyme is present in the cell in two fractions, one of which is easily extracted and the other fixed to insoluble constituents of the cell from which it can be liberated only with difficulty.

At 70° (Fig. 5), as at 37°, much activity is extracted very rapidly; for 1 hr. thereafter the rate of extraction falls, and later destruction of the

Fig. 5. Rate of extraction of enzyme at 70°. A suspension of 120 mg. of acetone powder in 4 ml. water was incubated at 70° and samples were withdrawn at intervals for the determination of decarboxylase activity in the extract.

enzyme sets in. The stability at 70° varies with different acetone powders. Thus one powder lost 69% of its activity after 1 hr. at 70°. The ease of extractability of the enzyme also varies (Table 2) for unknown reasons.

Table 2. Variation in extractability of the enzyme

The various acetone powders were incubated at 37° in aqueous suspension at a concentration of 5 mg./ml. for the times shown in the table. After incubation the suspensions were centrifuged and the decarboxylating activity of a suitable aliquot of the supernatant determined.

Incubation time (hr.) ...	Acetone powder preparation					
	A	B		C		D* E* F*
Initial activity extracted (%)	62	13	52	43	80	284 22 76
Initial Qco ₂	136	2300		2230		1065 1030 1210

* These suspensions contained capryl alcohol.

The amount of enzyme extracted is independent of the concentration of the acetone powder suspension within the range 1–30 mg./ml. With high concentrations, however, the centrifuging of residual insoluble material becomes difficult. A concentration of 15 mg. acetone powder/ml. seems to be the most suitable.

Purification of the enzyme

Adsorption on to alumina C γ . The first step in the purification was adsorption on to alumina C γ . Of the other adsorbing agents tried, kaolin adsorbed well at pH 4.0, and to some extent (30%), at pH 5.0, but not at reactions alkaline to this; Ca phosphate adsorbed 84% of the enzyme at pH 7.2 while kieselguhr adsorbed 78% at pH 4.0. Alumina C γ suspension (13–17 mg. alumina/ml.) possesses several advantages; at pH 5.0 and 6.0, it adsorbs 70–80% of the enzyme, but at pH 7.0 and above less than 6%. For adsorption, the extract was therefore brought to pH 5.0 by the addition of $\frac{1}{10}$ vol. of 2M acetate buffer. An extract derived from an acetone powder suspension of 5 mg./ml. requires $\frac{1}{10}$ vol. alumina suspension, but with stronger extracts more alumina is required, 0.3 vol. being sufficient up to 30 mg./ml.

Adsorption is very rapid though not instantaneous; 60% is adsorbed in less than 1 min., 89% in 5 min. and 93% in 10 min. The rate of elution of the enzyme is very similar. 10 min. was allowed for both processes.

Elution of the enzyme. The alumina was suspended in M/5 phosphate buffer at pH 7.0 and after 10 min. was centrifuged. For maximum elution the volume of eluent should be at least double the volume of alumina suspension used in the adsorption of the enzyme.

Precipitation by ammonium sulphate. Solid $(\text{NH}_4)_2\text{SO}_4$ was now added to the clear yellow eluate to give 80% saturation. The solid was used to keep the volume low: 75 g./100 ml. of solvent represents saturation, and other figures are based proportionally. The precipitate was separated by centrifuging and redissolved in a volume of distilled water equal to about 25% of that of the eluate. Fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ failed to separate out the enzyme consistently in any one fraction probably owing to variations in protein impurities. Accordingly it was decided to remove more of the inactive protein before attempting fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$.

Removal of inactive protein. The addition of $\frac{1}{10}$ vol. of 2M acetate buffer at pH 4.0 to the solution of the fraction precipitated by 80% sat. $(\text{NH}_4)_2\text{SO}_4$ was found to precipitate much of the inactive material; subsequent addition with rapid mixing of $\frac{1}{2}$ vol. of ethanol to the same solution precipitated a further quantity. More ethanol ($\frac{1}{2}$ vol.), however, inactivates the enzyme.

The enzyme could be recovered from the alcoholic solution by adsorption on to alumina C γ and elution with M/5 phosphate buffer of pH 7.0, using 0.3 vol. of alumina suspension and a volume of phosphate equal to the volume of the preparation before

ethanol treatment. For the adsorption with alumina the pH remained at 4.0.

Fractional precipitation with ammonium sulphate. After the above treatment it was found that most of the enzyme was consistently precipitated in the 50–60% sat. $(\text{NH}_4)_2\text{SO}_4$ fraction, and that this was also the purest fraction.

It should be pointed out that the degree of purification obtained in each stage varies considerably from one preparation to another but at the last stage the activity is never less than $Q_{\text{CO}_2}^c = 200,000$.

To carry purification further much time and starting material would be needed. From 6 to 9 g. of acetone-treated cells, which is the entire crop from 18 l. of medium, the total amount of protein in the enzyme preparation when an activity of 300,000 is reached, is of the order of 2–5 mg., of which 0.5–1.0 mg. is required for a reliable estimation of the C content. A few further treatments were, however, tried with variable results. Heating the preparation to 55° or even to 65° did not remove any inactive material; a second adsorption of the enzyme on to alumina C γ and elution with M/5 phosphate did not increase activity; concentration of the final preparation, and repetition of the ethanol precipitation at pH 4.0, resulted, in one case, in division of the enzyme between the ethanol precipitate and the solution when the precipitate had a slightly increased activity, but in another case most of the activity was lost.

Difficulties in centrifuging the enzyme precipitates. Satisfactory sedimentation of the enzyme at high concentrations of $(\text{NH}_4)_2\text{SO}_4$ (60% and above) presented difficulty. This was especially marked with the more highly purified preparations, and when a $Q_{\text{CO}_2}^c$ of 200,000–300,000 is reached the precipitate formed on 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ cannot be separated on the centrifuge at 3500 r.p.m. Such precipitates have to be filtered off through a G4 glass filter, a very slow process. The addition of a drop of capryl alcohol, followed by the thorough removal of dissolved air by evacuation, improves the sedimentation of the precipitates in the earlier stages of the purification, but fails in the final stage.

The final purification procedure. The details of one typical experiment on the purification of the enzyme are set out below:

(1) 5.2 g. of acetone-treated cells ($\Sigma \text{U.} = 4260$; $Q_{\text{CO}_2}^c = 2810$) were suspended in about 1 l. of distilled water and incubated at 37° for 4 hr. The suspension was centrifuged and 1030 ml. of a cloudy yellow extract were obtained ($\Sigma \text{U.} = 1725$; $Q_{\text{CO}_2}^c = 1180$).

(2) To the extract were added $\frac{1}{10}$ vol. of 2M acetate buffer of pH 5.0 and $\frac{1}{10}$ vol. of alumina C γ suspension (16.7 mg. alumina/ml.). After 10 min. the alumina was spun off and suspended in 203 ml. M/5 phosphate buffer of pH 7.0. After another

10 min. this was centrifuged and 203 ml. of a clear yellow solution obtained ($\Sigma.U. = 900$; $Q_{CO_2}^C = 7040$).

(3) To the eluate were added 120 g. $(NH_4)_2SO_4$ giving 80% saturation; the centrifuged precipitate gave a yellow solution in 37.5 ml. distilled water ($\Sigma.U. = 710$; $Q_{CO_2}^C = 45,000$).

(4) To this solution were added $\frac{1}{20}$ vol. 2M acetate buffer at pH 4.0 and then $\frac{1}{5}$ vol. ethanol, with vigorous shaking. After a few minutes the precipitate was spun off and 11 ml. of alumina C γ suspension (strength as above) were added to the centrifugate; after 10 min. the alumina was spun off and the enzyme eluted for 10 min. with 37.5 ml. M/5 phosphate buffer at pH 7.0. A clear, slightly yellow solution was obtained ($\Sigma.U. = 539$; $Q_{CO_2}^C = 85,000$).

(5) To this eluate were added 13.6 g. $(NH_4)_2SO_4$ giving 50% saturation; the precipitate was spun off and discarded. The centrifugate contained the enzyme ($\Sigma.U. = 440$; $Q_{CO_2}^C = 172,000$).

(6) To the centrifugate 2.73 g. $(NH_4)_2SO_4$ were added giving 60% saturation. The precipitate was filtered off and dissolved in 5.2 ml. distilled water; the solution contained 181 units of $Q_{CO_2}^C = 330,000$. This was the final preparation (Table 3). The most

Table 3. Purification of the enzyme

Treatment	Enzyme units	$Q_{CO_2}^C$
(1) Original acetone powder	4260	2,810
(2) Aqueous extract	1725	1,180
(3) Adsorption on to alumina and elution with M/5 phosphate pH 7.0	900	7,040
(4) Precipitation by 80% saturation with $(NH_4)_2SO_4$	710	45,000
(5) Precipitation by $\frac{1}{5}$ vol. ethanol at pH 4.0 followed by adsorption on to alumina and elution with phosphate	539	85,000
(6) Precipitation by $(NH_4)_2SO_4$ between the limits 50–60% saturation	181	330,000

active preparation so far obtained had an activity of 404,000 (but see below on the dissociation of the enzyme).

Properties of the purified enzyme

Strong solutions of the purest enzyme yet obtained are very slightly yellow, perhaps owing to impurities, since yellow pigments are eliminated at almost every stage of the purification. The ultra-violet absorption spectrum determined in the Spekker spectrophotometer shows a typical protein absorption curve having a maximum at 275 m μ and $\log I_0/I$ of 2.8 at this wave-length for a solution containing 0.533 mg. C/ml. (about 1 mg. protein). All the following experiments were carried out with the enzyme preparation of $Q_{CO_2}^C = 404,000$.

(1) *Reaction velocity: effect of pH.* This was determined in M/5 acetate buffer. The optimum pH is seen to lie at 4.9–5.0 (Fig. 6).

Fig. 6. Effect of pH on the rate of decarboxylation by the purified enzyme. Manometers contained 0.5 ml. enzyme solution, 0.3 ml. 2M acetate buffer of the required pH, 0.5 ml. M/2 Na acetoacetate; total vol. 3.0 ml.; temp. 37.5°.

(2) *Effect of substrate concentration.* Fig. 7 shows that the affinity of the enzyme for its substrate is fairly low, a concentration of 0.05M acetoacetic acid being required to saturate the enzyme, the half-speed concentration being 0.008M.

Fig. 7. Effect of substrate concentration on the rate of decarboxylation by the purified enzyme. Manometers contained 0.5 ml. enzyme solution, 0.3 ml. 2M acetate buffer pH 5.0, M/2 Na acetoacetate to give the required concentration; total vol. 3.0 ml.; temp. 37.5°.

(3) *Effect of temperature.* The temperature coefficient increases slightly with rise in temperature (Fig. 8). For the range 20–30° the $Q_{10} = 1.82$ and for 30–40° the $Q_{10} = 2.16$.

Fig. 9 shows the effect of 5 min. exposure to various temperatures, the activity being determined at 37.5°. Little destruction occurs up to 70°; above 70° the enzyme is rapidly inactivated, being almost completely destroyed in 5 min. at 85°. Inactivation

at 60° occurs at a fairly steady rate (Fig. 10). There was no detectable loss in activity of an aqueous solution that had been stored for 5 weeks in the ice-chest at 4°.

Fig. 8. Effect of temperature on the rate of decarboxylation by the purified enzyme. Manometers contained 0.5 ml. enzyme solution, 0.3 ml. 2 *M* acetate buffer pH 5.0, 0.5 ml. *M*/2 Na acetoacetate; total vol. 3.0 ml.; temp. as indicated.

Fig. 9. Effect of temperature on the stability of the enzyme. 1 ml. enzyme preparation was heated for 5 min. at the temperatures shown in the figure; 0.5 ml. was then used for the determination of activity under the optimum conditions.

(4) *Effect of inhibitors.* The effects of well-known enzyme inhibitors are given in Table 4. Unlike yeast carboxylase [Green, Herbert & Subrahmanyam, 1941] the enzyme is comparatively insensitive to Cu, though very sensitive to Ag and Hg; KCN is a

fairly powerful inhibitor of it and NaN_3 inhibits it in high concentrations. α -Keto-acids inhibit it to

Fig. 10. Effect of time on the activity of the enzyme incubated at 60°. 4 ml. of the enzyme preparation were incubated at 60° and samples (0.5 ml.) were withdrawn at intervals for determination of decarboxylase activity.

varying degrees and outstanding in this class is acetopyruvic acid which causes 65 % inhibition at a concentration of $M \times 10^{-6}$. The structural relationships between this substance and acetoacetic

Table 4. *Effect of inhibitors on decarboxylase activity*

Inhibitor	Conc. <i>M</i>	Inhibition %
Heavy metals:		
$FeCl_3$	10^{-2}	45
$CuSO_4$	2×10^{-2}	41
$AgNO_3$	10^{-5}	90
$HgCl_2$	10^{-5}	100
General:		
Capryl alcohol	Satd. sol.	0
Na_2S	10^{-1}	0
Urethane	10^{-1}	16
Toluene	Satd. sol.	47
NaN_3	10^{-2}	53
Iodoacetic acid	10^{-2}	63
KCN	10^{-4}	82
Competitive:		
Acetone	1	10
Methyl ethyl ketone	1	17
Acetone dicarboxylic acid	5×10^{-2}	22
Pyruvic acid	5×10^{-2}	64
α -Ketoglutaric acid	5×10^{-2}	70
Ethyl acetoacetate	10^{-1}	72
Malonic acid	10^{-1}	76
Oxaloacetic acid	10^{-2}	44
Acetopyruvic acid	10^{-6}	65

acid suggest that the inhibition is of a competitive kind, acetopyruvic acid being the most closely related and also the most powerfully inhibitory. The marked inhibition of acetoacetic acid decarboxylase by low concentrations of acetopyruvic acid disposes entirely of the theory that this substance is an intermediate in the formation of acetone.

(5) *Specificity of the enzyme.* Table 5 shows that the enzyme is specific for the decarboxylation of acetoacetic acid within the range of substances tested.

Table 5. *Specificity of the enzyme*

Substrate	Conc. M	CO ₂ μl./5 min.
Acetoacetic acid	0.08	138
Ethyl acetoacetate	0.1	0
Pyruvic acid	0.05	0
Acetopyruvic acid	0.05	0
Oxaloacetic acid	0.01	0
α-Ketoglutaric acid	0.05	0
Acetone dicarboxylic acid	0.05	0

All the determinations were made in *M*/5 acetate pH 5.0.

(6) *Effect of enzyme concentration.* Fig. 11 (curve 1) shows that the reaction velocity increases more rapidly with increasing enzyme concentration than would be expected on the basis of a linear

Fig. 11. Effect of enzyme concentration on the rate of decarboxylation. (1) without yeast extract, (2) in presence of yeast extract. Manometers contained 0.3 ml. 2*M* acetate buffer pH 5.0, 0.5 ml. *M*/2 Na acetoacetate, 0.5 ml. of a boiling-water extract of dried brewer's yeast in one series, and enzyme solution as shown in the figure; total vol. 3.0 ml.; temp. 37.5°.

relationship. This suggests that at low concentrations the enzyme may dissociate into a specific protein and a coenzyme, only the undissociated enzyme being catalytically active. That this is the case is shown by curve 2 where the reaction velocity

has been determined in the presence of a boiling-water extract of dried brewer's yeast; here the relationship between enzyme concentration and reaction velocity is linear. Presumably the extract contained the coenzyme in sufficient concentration to counteract the dissociation of the enzyme.

The concentration of enzyme in the region where dissociation is greatest is only about 0.7 μg./ml. and it is in fact only because of the high activity of the enzyme that the dissociation can be observed. Assuming that the enzyme is pure it can be calculated from the curves of Fig. 11 that the dissociation constant for the enzyme is very approximately 1.55×10^{-8} mol./l. This may be compared with the value of 2.5×10^{-7} mol./l. for *d*-amino-acid oxidase [Warburg & Christian, 1938a].

Attempts to separate enzyme into protein and coenzyme

The dissociation of the enzyme in low concentrations should make it possible to separate the protein from the coenzyme by dialysis; all attempts so far, however, have been unsuccessful; dialysis for 24 hr. at 37° of 30 ml. of a solution containing about 1 μg. enzyme/ml. against 4 l. of *M*/5 acetate buffer at pH 5.0 effected no change in the activity of the enzyme, these conditions being precisely those under which dissociation is observed in the manometer.

Precipitation with ammoniacal (NH₄)₂SO₄, which reversibly dissociates yeast carboxylase [Green *et al.* 1941] does not so dissociate acetoacetic acid decarboxylase. At room temperature, at 45° for 5 min., at 55° for 5 min. and at 0° for 20–40 hr., there is a varying loss of activity, but in no case can the activity be restored by adding a boiled suspension of acetone powder or of dried yeast. The same is true after precipitation by (NH₄)₂SO₄ in acid solution by the method of Warburg & Christian [1938b] as described by Weil-Malherbe [1939], while increase in the final acid concentration up to *M*/22 HCl results in a 50% loss of activity which also cannot be restored. Adjustment of the enzyme solution to pH 2 or pH 3 followed by precipitation with 10 vol. acetone results in complete loss of activity. Finally, 2 ml. of enzyme solution were dialysed for 22 hr. at 17° against 800 ml. of a solution containing 9 parts 70% sat. (NH₄)₂SO₄ solution and 1 part conc. NH₃ solution, and this was followed by dialysis for 1 hr. against running tap water. The enzyme was then found to have lost 76% of its activity, which loss could not be restored by adding boiled cells. It must be assumed in view of these failures to restore the activity of the enzyme that all the above procedures have not caused the enzyme to dissociate but have merely inactivated it.

The nature of the coenzyme

Despite the failure to split the enzyme reversibly into a coenzyme and a specific protein, some information as to the identity of the coenzyme has been obtained by studying the reversal of the dissociation that occurs at low enzyme concentrations. As has been shown, yeast extract and an extract of acetone-treated cells can counteract the dissociation, hence the ability of other substances to replace the extracts was investigated. The compounds tested were cocarboxylase, thiamin monophosphate, thiamin, muscle adenylic acid, cozymase (60–70 % pure), an old preparation of diaphorase containing a considerable proportion as free flavin and, finally,

riboflavin had already been shown to be inactive with fair certainty. There remained the first three possibilities. Table 7 shows the effect of boiling, and of hydrolysis in acid and alkali, on the activity of the diaphorase preparation. It is seen that boiling and acid hydrolysis do not alter the activity, but that hydrolysis with alkali causes some inactivation. The first result shows that diaphorase itself cannot be the coenzyme, while the result of acid hydrolysis rules out riboflavin adenine dinucleotide since, under the conditions used, this substance would be hydrolysed into adenylic acid and riboflavin phosphate [Abraham, 1939]. Riboflavin phosphate, however, is not split under these conditions [Kühn & Rudy, 1936]. Unfortunately it has been impossible to

Table 6. *Inhibition of dissociation by various substances*

The manometers contained 0.3 ml. 2 M acetate buffer pH 5.0, 0.5 ml. *M/2* acetoacetic acid, 0.1 ml. enzyme solution containing about 0.7 μ g. protein; the total vol. was 3.0 ml. Temp. 37.5°. The diaphorase preparation contained total flavin equivalent to about 20 μ g. lactoflavin/ml.

Substance tested

μ l. CO ₂ /5 min.	Substance tested												
	Nil	10% yeast extract 0.5 ml.	1% extract of acetone-treated cells 0.5 ml.	Co-carbo- xylase 50 μ g.	Thiamin mono- phos- phate 40 μ g.	Thiamin 100 μ g.	Muscle adenylic acid 100 μ g.	Co- zymase 100 μ g.	Lactoflavin		Diaphorase prep.		
									61 μ g.	6 μ g.	0.1 ml.	0.01 ml.	0.001 ml.
	18	54	53	21	15	22	23	43	38	19	51	48	30

riboflavin. The results (Table 6) show that adenylic acid and the thiamin derivatives are totally inactive, while cozymase and riboflavin exhibit sub-maximal activity; the diaphorase preparation on the other hand is maximally active at a concentration of 0.0033 ml./ml. (about 0.07 μ g. riboflavin/ml.). It is probable that the activity of riboflavin and of the cozymase preparation is due to an impurity, since even large amounts of these substances do not show maximum activity.

The active diaphorase preparation was originally about 90 % pure. There were therefore five possibilities, that the active substance was (a) diaphorase itself, (b) the prosthetic group of diaphorase, riboflavin adenine dinucleotide, (c) riboflavin phosphate, (d) riboflavin, and (e) an impurity in the diaphorase preparation. In view of the small amount of the diaphorase preparation required for maximum activity the last possibility did not seem likely, although it has not yet been excluded, while

complete these experiments at the present time, but it seems possible that riboflavin phosphate is actually the coenzyme; the effect of hydrolysis with alkali is evidence in support of this idea.

SUMMARY

1. The purification and properties of acetoacetic acid decarboxylase of *Cl. acetobutylicum* have been described.

2. The purified enzyme is quite stable at ordinary temperatures; it is optimally active at pH 5.0 and is specific for acetoacetic acid.

3. In dilute solution the enzyme is dissociated but it has not been possible to separate the component parts.

4. The dissociation can be prevented by a preparation of diaphorase at a concentration equivalent to 0.07 μ g. riboflavin/ml. Riboflavin is inactive. Mild acid hydrolysis (*N/20* HCl for 60 min. at 96°) does not affect this activity of the diaphorase preparation.

5. It is tentatively suggested that riboflavin phosphate may be the prosthetic group of the enzyme.

Table 7. *Effect of boiling and of hydrolysis by acid and alkali on the activity of the diaphorase preparation*

The manometers contained the equivalent of 0.1 ml. of the diaphorase preparation; the other contents were as described in Table 6.

Treatment of diaphorase...	None	Heated at 100° for 5 min.	Heated at 96° for 60 min. in <i>N/20</i> HCl	Boiled for ½ min. in <i>N/10</i> NaOH	Control, no diaphorase
μ l. CO ₂ /5 min.	52	50	46	36	18

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The Influence of Dietary Fat of Varying Unsaturation on the Component Acids of Cow Milk Fats

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In feeding trials in which the basal ration of a group of dairy cows was supplemented by 1 lb./day of various fatty oils, Brown, Dustman & Weakley [1941] observed that the iodine value (i.v.) of the milk fat was altered in different ways by different fats, a feeding period of about 3 weeks being necessary before the extent of the change in i.v. reached its maximum. Coconut oil caused a slight reduction of 4 or 5 units in the i.v. of the milk fat, both crude and refined soya bean oil (i.v. ca. 127) increased it greatly (by over 50 %), and hydrogenated soya bean oil (i.v. 17) increased it slightly (by about 5 or 6 units), as well as having a pronounced laxative action on the cows. These authors ascribed the slight increase in the unsaturation caused by feeding the hydrogenated oil to desaturation of the fat in the ration, an assumption for which there seems to be no evidence.

Similar alterations in the unsaturation of cow milk fats (as indicated by their i.v.'s alone), which result from administration of specific fats in the diet, have been published. Thus, Holland & Buckley [1918; 1923] who fed cows on a basal ration, and on the basal ration supplemented by $\frac{1}{2}$ lb. daily of coconut, groundnut, maize or soya bean oils, observed a slight fall in i.v. with the coconut oil diet, but marked increases (35–45 %) with the three liquid fats. Again, Hill & Palmer [1938] found that the inclusion of maize, cottonseed or linseed oils in the diet increased the i.v.'s, and slightly diminished the Reichert-Meissl values of the corresponding milk fats; tallow slightly increased the milk fat i.v., while butter slightly decreased it, and coconut oil decreased it by 4 or 5 units. Such observations clearly only measure changes in the mean unsaturation of the milk fats, and do not indicate which of

the component acids has significantly altered in amount. Thus, whilst oleic acid is the chief unsaturated acid, at least six others may be present in small proportions in cow milk fat, together with nine saturated acids (of which palmitic acid is the chief, though there are appreciable amounts of butyric, myristic and stearic acids). For a clear picture of milk-fat component acids a detailed analysis of the proportions of each acid is necessary.

Few detailed examinations have been made of the effect of supplementary fats in the cow's diet. Hilditch & Sleightholme [1930] examined milk fats from cows receiving coconut cake or soya bean cake in addition to their normal winter ration; the daily ration of cake (7 lb. coconut, 5 lb. soya bean) could not have contained more (and possibly much less) than 6–7 oz. of fatty oil. The milk fat from cows receiving coconut cake contained less oleic and more lauric and myristic acids than normal, whilst that from animals receiving soya bean cake had somewhat less oleic but the normal amount of octadecadienoic acid, and was otherwise similar to the control milk fats. Hilditch & Thompson [1936] observed similar effects when 4 oz. of linseed, rape or cod-liver oil were added to the daily basal ration of cows. With linseed or rape oils, the component acids differed little, but the proportion of palmitic acid was reduced and that of oleic somewhat increased; with linseed oil, no linolenic acid was present, but the proportion (5.9 % by wt.) of octadecadienoic acids was higher than usual (ca. 3 %), whilst with rape oil the octadecadienoic acids were normal (3.3 %) but there was a similar amount of erucic acid. With cod-liver oil the results were more striking: the milk fat here contained an unusually large amount (5.8 %) of highly unsaturated acids