- Cole, S. W., Hanes, C. S., Jackson, H. A. F. & Loughman, B. C., quoted by Bell, D. J. (1955). In *Modern Methods of Plant Analysis*, vol. 2, p. 1. Ed. by Paech, K. & Tracey, M. V. Berlin, Göttingen, Heidelberg: Springer-Verlag.
- Edelman, J., Ginsburg, V. & Hassid, W. Z. (1955). *J. biol. Chem.* **213**, 843.
- Euler, H. von, Schlenk, F., Heiwinkel, H. & Höberg, B. (1938). *Hoppe-Seyl. Z.* **256**, 208.
- Kornberg, A. (1951). *Phosphorus Metabolism*, vol. 1, p. 392. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
- Leloir, L. F. & Cardini, C. E. (1953). *J. Amer. chem. Soc.* **75**, 6084.
- Leloir, L. F. & Cardini, C. E. (1955). *J. biol. Chem.* **214**, 157.
- Leonard, O. A. (1939). *Amer. J. Bot.* **26**, 475.
- Munch-Petersen, A., Kalckar, H. M., Cutolo, E. & Smith, E. E. B. (1953). *Nature, Lond.*, **172**, 1036.
- Nelson, J. M. & Auchincloss, R. (1933). *J. Amer. chem. Soc.* **55**, 3769.
- Nurmia, N. (1935). *Ann. Acad. Sci. fenn. A*, **44**, no. 8.
- Porter, H. K. & May, L. H. (1955). *J. exp. Bot.* **6**, 43.
- Putman, E. W. & Hassid, W. Z. (1954). *J. biol. Chem.* **207**, 885.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- Turner, J. F. (1953). *Nature, Lond.*, **172**, 1149.
- Turner, J. F. (1954). *Nature, Lond.*, **174**, 692.
- Vittorio, P. V., Krotkov, G. & Reed, G. B. (1954). *Canad. J. Bot.* **32**, 369.

Formation of 3-Hydroxybutan-2-one and Butane-2:3-diol by Pig-heart Preparations and its Relationship to Oxidation of Pyruvate

By J. DAWSON, R. P. HULLIN AND MARJORIE WALKER
Department of Biochemistry, University of Leeds

(Received 21 November 1956)

3-Hydroxybutan-2-one (acetoin) appears in mammalian systems, plants and yeasts to be formed by a similar mechanism. Pyruvate is decarboxylated to an active form of acetaldehyde, probably an acetaldehyde-diphosphothiamine complex, $[\text{CH}_3 \cdot \text{CHO}]$, which condenses with free acetaldehyde:

All the evidence suggests that only pyruvic oxidase is required for the condensation (Berg & Westerfeld, 1944; Juni, 1952; Singer & Pensky, 1952; Langenbeck & Faust, 1953).

This mechanism differs from the one most commonly observed in bacteria, i.e. formation of a complex followed by condensation with pyruvate to form α -acetyl-lactate, which is then decarboxylated to acetoin (Slade & Werkman, 1943; Krampitz, 1948; Strecker & Ochoa, 1954):

Decarboxylation of pyruvic acid is common to both systems and also to the system concerned with acetyl-coenzyme A (acetyl-CoA) formation. The main difference between the three systems is the acceptor for the activated acetaldehyde produced. In bacterial production of acetoin pyruvic acid is the acceptor, with the intermediate production of α -acetyl-lactic acid; in plants, yeasts and mammals the acceptor is free acetaldehyde, and for formation of acetyl-CoA α -lipoic acid acts as an intermediate acceptor (Schweet & Cheslock, 1952; Stotz, 1954).

Formation of activated acetaldehyde from pyruvate probably occurs in the same manner in all organisms, though its further metabolism is influenced by the availability of other enzymes and cofactors. This was demonstrated by Stern, Shapiro, Stadtman & Ochoa (1951) and by Korkes, del Campillo, Gunsalus & Ochoa (1951), who showed that pyruvic oxidases from mammalian and bacterial sources were interchangeable. The former workers discovered that soluble enzyme preparations from *Escherichia coli* and *Streptococcus faecalis* in the presence of diphosphopyridine nucleotide (DPN) catalysed the reaction:

In the presence of bacterial phosphotransacetylase and coenzyme A (CoA) the following reaction occurred:

In the absence of phosphate these reactions could not occur, but, if phosphate and bacterial phosphotransacetylase were replaced by oxaloacetate and mammalian condensing enzyme, reaction (3) occurred:

Korkes *et al.* (1951) prepared an enzyme from pig-heart muscle which, supplied with the appropriate cofactors, would synthesize citrate from pyruvate and oxaloacetate in the presence of condensing

enzyme (also prepared from pig heart). If oxaloacetate and condensing enzyme were replaced by phosphate and bacterial transacetylase, then acetyl phosphate was produced, as in bacterial systems.

In the work with pig-heart enzyme and bacterial phosphotransacetylase, yields of acetate and lactate were slightly less than the theoretical. The authors suggested that the slight excess of pyruvate utilization might be due to formation of acetoin, although this substance was not estimated. With the complete animal system it was not possible to estimate utilization owing to the interference caused by oxaloacetate, but it was possible that formation of acetoin might occur there also. A similar preparation was therefore made and its acetoin-forming ability was investigated.

The method of extraction used by Korkes *et al.* (1951) was modified slightly and yielded a preparation which produced acetoin with pyruvate as substrate. This was investigated under various conditions in order to elucidate the relative importance of various factors concerned in production of acetoin in mammalian systems.

METHODS

Production of carbon dioxide was estimated by the Warburg manometric technique described by Dixon (1934), pyruvic acid by the method of Friedemann & Haugen (1943), 3-hydroxybutan-2-one (acetoin) and butane-2:3-diol by the method of Happold & Spencer (1952); acetic acid was estimated by a modification of the method of McLendon (1944), with the apparatus described by Markham (1942) and removal of pyruvate interference as suggested by Weil-Malherbe (1937); citric acid was estimated by the method of Taylor (1953) and nitrogen by a micro-Kjeldahl method with the apparatus of Markham (1942).

Lithium pyruvate was prepared by a method described by Wendel (1931), and condensing enzyme by the method of Ochoa, Stern & Schneider (1951). Lactic dehydrogenase and thioctic acid were obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

Preparation of acetoin-forming enzyme. Two fresh pig hearts stripped of fat and connective tissue were cooled to about 4° and minced in a coarse mincing machine. The mince was washed four times with ice-cold water, strained through white lint, separated into 80 g. portions, frozen at -18° and stored for a period not exceeding 5 weeks.

The preparation of active enzyme extracts from the mince was carried out at 0-4° by the following procedure. Mince (80 g.) was partially thawed and homogenized with 280 ml. of 0.02M-potassium phosphate buffer, pH 7.0, for 6 min. The mixture was allowed to stand for 30 min., then centrifuged at 2900 g for 20 min. The residue was extracted in the same way with 90 ml. of buffer.

The combined supernatants were made 30% saturated with ammonium sulphate and allowed to stand for 20 min. After centrifuging at 2900 g the precipitate was taken up in 20 ml. of 0.02M-potassium phosphate buffer, pH 7.0. After dialysis overnight against the same buffer, the solution was centrifuged to remove any precipitate and was then ready for immediate use.

Constitution of buffer solutions. Unless stated otherwise these were as follows: pH 6.2, 1.9 vol. of 0.2M-Na₂HPO₄ + 8.1 vol. of 0.2M-KH₂PO₄; pH 7.4, 8.15 vol. of 0.2M-K₂HPO₄ + 1.85 vol. of 0.2M-KH₂PO₄.

General procedure. Enzyme solution, buffer, diphosphothiamine and magnesium chloride were placed in a Warburg flask. Cofactors under investigation were added to the mixture in selected flasks. The substrate(s) were placed in a side arm and added after equilibration. When cofactors were added, the air in the flasks was replaced by N₂. At pH 6.2 there was no retention of carbon dioxide in the course of the experiment. At pH 7.4 retention occurred, so acid was tipped in from a second side arm to obtain corrected figures for production of carbon dioxide. The reaction was allowed to proceed for 1 hr. at 37°, then terminated by the addition of protein precipitants.

The enzyme preparation, though far from pure, was fairly constant with regard to its ability to form acetoin. Assayed under standard conditions at pH 6.2 in the presence of 10 μmoles of lithium pyruvate, 100 μg. of diphosphothiamine and 4 μmoles of magnesium chloride in a total volume of 4 ml., a pyruvate:carbon dioxide:acetoin + butane-2:3-diol ratio of approx. 2:2:1 was obtained from several preparations. The 2 ml. of enzyme solution used in each Warburg flask contained approx. 2 mg. of nitrogen. The results obtained are means of duplicate or triplicate estimations.

RESULTS

The pH-activity curve of the enzyme preparation is shown in Fig. 1. As this study was concerned with the relationship of the formation of 3-hydroxybutan-2-one (acetoin) and of citrate by this preparation it

Fig. 1. Activity-pH curve for production of acetoin from pyruvic acid. The conditions were as described in the text.

Table 1. *Effect of pH and concentration of Na⁺ and K⁺ ions on production of acetoin and butane-2:3-diol*

Enzyme (2 ml.), 4 μ moles of MgCl₂, 100 μ g. of diphosphothiamine, 50 μ moles of lithium pyruvate and 1.2 ml. of 0.2M-phosphate buffer were incubated at 37° in air. Results are expressed as μ moles/mg. of nitrogen/hr.

Na/K	...	...	...	...	pH 6.2		pH 7.4	
					0.5	8.8	0.5	8.8
Carbon dioxide produced					1.00	0.85	0.65	0.35
Pyruvate utilized					1.20	1.05	1.00	0.75
Acetoin and butane-2:3-diol produced					0.50	0.45	0.35	0.25

Fig. 2. Production of acetoin and butane-2:3-diol from pyruvic acid at pH 6.2. The conditions were as described in the text.

Table 2. *Effect of concentration of acetaldehyde on production of acetoin and butane-2:3-diol from a constant amount of pyruvic acid*

Enzyme (2 ml.), 4 μ moles of MgCl₂, 100 μ g. of diphosphothiamine, 10 μ moles of lithium pyruvate, 200 μ moles of phosphate buffer, pH 6.2, and varying amounts of acetaldehyde were incubated at 37° in air.

Acetaldehyde (μ moles)	Amounts produced (μ moles/mg. of nitrogen/hr.)		
	Acetoin	Butane-2:3-diol	Carbon dioxide
0	0	0.40	0.50
25	0.45	0.90	1.10
50	0.75	1.65	1.70
75	1.55	1.70	2.60
100	1.70	2.10	3.05
125	1.50	2.00	2.90
150	1.65	2.00	3.50
175	2.35	1.05	4.05
200	2.30	0.55	3.40

was important to determine factors which would increase the yield of acetoin at pH 7.4, which is the optimum pH for formation of citrate. Alterations

in the sodium:potassium ratio were made by using potassium chloride to increase the concentration of potassium in the buffers with a sodium:potassium ratio of 8.8. Table 1 demonstrates the effect of changing the ionic ratio at a pH of 6.2 and 7.4. Mean values are given. At pH 6.2 a change in the sodium:potassium ratio had no significant effect on production of acetoin and butane-2:3-diol. At pH 7.4, the production of these compounds was increased at the lower sodium:potassium ratio ($t=6.5$, $P<0.001$). In subsequent investigations at pH 7.4 a potassium buffer was used.

The effect of increasing pyruvate concentration on the production of acetoin and butane-2:3-diol is shown in Fig. 2.

The K_m of the enzyme for production of acetoin and butane-2:3-diol with pyruvate as substrate was $0.62 \times 10^{-5} M$.

A mixture of acetaldehyde and pyruvate was also a good substrate for the enzyme. In Table 2 results are given for production of acetoin and butane-2:3-diol at pH 6.2 from 10 μ moles of pyruvate and varying quantities of acetaldehyde. The K_m of the production of acetoin and butane-2:3-diol for this system was $1.1 \times 10^{-4} M$; a value of $3.4 \times 10^{-4} M$ was found by Juni (1952).

The maximum production of acetoin and butane-2:3-diol in this experiment is equivalent to a disappearance of 95% of the pyruvic acid, assuming a pyruvic acid:(acetoin + butane-2:3-diol) ratio of 1; the mean carbon dioxide:(acetoin + butane-2:3-diol) ratio was 0.78. This represents a reduced production of carbon dioxide for the amount of acetoin and butane-2:3-diol produced. At pH 7.4, with 10 μ moles of pyruvic acid + 100 μ moles of acetaldehyde the enzyme produced 4.04 μ moles of acetoin and butane-2:3-diol/mg. of nitrogen/hr. Thus, while pH has a marked effect on the production of these compounds from pyruvate as sole substrate, it has no significant effect when free acetaldehyde is added to the reaction mixture.

A different effect is observed if the acetaldehyde concentration is kept constant and increasing amounts of pyruvate are added. This is illustrated in Table 3, which gives the production of acetoin and butane-2:3-diol from 20 μ moles of acetaldehyde and varying amounts of pyruvate at pH 6.2.

The mean CO_2 /acetoin + butane-2:3-diol ratio when the substrate was a mixture of pyruvate and acetaldehyde was 0.33, indicating a marked decrease in production of carbon dioxide, which is much lower than when the acetaldehyde concentration was varied with a constant amount (10 μ moles) of pyruvate present. In addition, the production of acetoin and butane-2:3-diol was inhibited as the concentration of pyruvic acid increased. If it is assumed that the production of carbon dioxide is an index of the production of acetoin and butane-2:3-diol due to formation of 'active acetaldehyde' by decarboxylation of pyruvate, then in the range 10–50 μ moles of pyruvate a mean production of 1.43 μ moles of acetoin and butane-2:3-diol/mg. of nitrogen/hr. is due to some other mechanism. Only a concentration of pyruvate of 5 μ moles gave rise to carbon dioxide and production of acetoin and butane-2:3-diol consistent with the hypothesis that pyruvate is decarboxylated to give 'active acetaldehyde', which then condenses with free acetaldehyde to give acetoin. At higher concentrations of pyruvate some other mechanism must also operate.

Table 4 demonstrates that oxaloacetate can be used instead of pyruvate as substrate; 30 μ moles of oxaloacetate were used in each case and the concentration of acetaldehyde was varied as shown. Oxaloacetate acts by decarboxylation to give pyruvate, which is further decarboxylated to give 'active acetaldehyde'.

Juni & Heym (1956) demonstrated that, when the substrate was pyruvate alone, vigorous aeration of the reaction mixture with nitrogen would remove free acetaldehyde and decrease the production of acetoin. The use of dimedone as an aldehyde-trapping reagent in the reaction mixture might be expected to yield similar results. Table 5 demonstrates the effects observed.

Dimedone has a stimulating effect on the production of acetoin and butane-2:3-diol from pyruvate. In the presence of acetaldehyde there is no evidence of any inhibitory effect; thus if

dimedone does trap the acetaldehyde under these conditions, it is still available for participation in the reactions producing acetoin and butane-2:3-diol.

Table 3. *Effect of concentration of pyruvate on production of acetoin and butane-2:3-diol with a constant acetaldehyde level*

Enzyme (2 ml.), 4 μ moles of magnesium chloride, 100 μ g. of diphosphothiamine, 20 μ moles of acetaldehyde, 200 μ moles of phosphate buffer, pH 6.2 and varying amounts of lithium pyruvate were incubated at 37° in air.

Pyruvate (μ moles)	Amounts produced (μ moles/mg. of nitrogen/hr.)		
	Acetoin	Butane-2:3-diol	Carbon dioxide
0	0.02	0.05	0
5	0.65	1.65	2.20
10	0.65	1.80	1.30
15	0.65	1.40	0.50
20	0.60	1.50	0.45
25	0.55	1.30	0.25
30	0.40	1.05	0.10
40	0.35	1.15	0.05
50	0.35	1.15	0.60
75	0.40	1.05	0.35

Table 4. *Oxaloacetic acid as a substrate for formation of acetoin and butane-2:3-diol*

Enzyme (2 ml.), 4 μ moles of magnesium chloride, 100 μ g. of diphosphothiamine, 30 μ moles of oxaloacetic acid, 200 μ moles of phosphate buffer, pH 6.2, and varying amounts of acetaldehyde were incubated at 37° in air.

Acetaldehyde (μ moles)	Amounts produced (μ moles/mg. of nitrogen/hr.)	
	Acetoin	Butane-2:3-diol
0	0.35	0.45
20	0.75	1.70
40	1.70	2.35
60	1.40	2.65
90	1.60	3.70

Table 5. *Effect of dimedone on production of acetoin and butane-2:3-diol from pyruvic acid*

Reaction mixture: 2 ml. of enzyme, 4 μ moles of magnesium chloride, 100 μ g. of diphosphothiamine, lithium pyruvate (10 μ moles), acetaldehyde (10 μ moles) and dimedone (1.5 ml. of 0.7% in 0.2 M-phosphate buffer, pH 6.2) were added where indicated. The mixture was incubated in air at 37°.

Substrate	Amounts produced (μ moles/mg. of nitrogen/hr.)		Carbon dioxide Acetoin and butane-2:3-diol
	Acetoin and butane-2:3-diol	Carbon dioxide	
Pyruvate	0.30	0.65	2.1
Pyruvate + dimedone	0.65	1.37	2.0
Pyruvate + acetaldehyde	1.30	—	—
Pyruvate + dimedone + acetaldehyde	1.65	1.06	0.7
Acetaldehyde + dimedone	0.15	—	—

Investigation of the effects of cofactors required for production of citrate on the production of acetoin and butane-2:3-diol from pyruvate

Although Mg^{2+} ions were essential for the production of acetoin and butane-2:3-diol from pyruvic acid, increasing the amount of Mg^{2+} ions above 2 μ moles did not affect the yields.

The effects of increasing the concentration of Mg^{2+} and Mn^{2+} ions on production of acetoin and butane-2:3-diol from a mixture of pyruvate and acetaldehyde are indicated in Table 6.

Increasing the concentration of Mg^{2+} ions increased the production of acetoin and butane-2:3-diol. Mn^{2+} ions in smaller concentration produced the same effect when added to a basal level of Mg^{2+} ions. There is, however, an excess of production of acetoin and butane-2:3-diol above that which would be expected from the values of pyruvate utilization. In part the stimulatory effect of Mn^{2+} ions involves this excess of production.

Other cofactors which were tested either separately or together were diphosphopyridine nucleotide (DPN), lactic dehydrogenase, coenzyme A, cysteine and condensing enzyme. These were without effect on the production of acetoin and butane-2:3-diol. From citrate estimations it was clear that our enzyme preparation contained optimum amounts of condensing enzyme and also sufficient coenzyme A.

Table 7 presents the results obtained by using as substrate a mixture of 10 μ moles of pyruvate and 100 μ moles of acetaldehyde with the addition of all the cofactors listed above, and, in some cases, oxaloacetate.

The stimulating effect of oxaloacetate on the production of acetoin and butane-2:3-diol is considered to be due to its increasing the effective concentration of pyruvate and the stimulating effect of the cofactor addition is attributed to the concentration of manganese. The addition of cofactors not only led to the production of citrate but also caused an increase in the production of acetoin and butane-2:3-diol.

Thioctic acid might be present in our enzyme system in suboptimum amounts, although a similar enzyme produced by Korkes *et al.* (1951) had optimum amounts. By the use of pyruvate (50 μ moles), oxaloacetate (30 μ moles) and cofactor additions as in Table 7, the effect of thioctic acid on production of acetoin and butane-2:3-diol and formation of citrate was investigated. Table 8 shows the results obtained.

Thioctic acid markedly stimulated formation of citrate without appreciably altering the production of acetoin and butane-2:3-diol.

When acetaldehyde (100 μ moles) was added to the reaction mixture in the presence of thioctic acid (100 μ g.) the results shown in Table 9 were obtained.

Table 6. *Effect of Mg^{2+} and Mn^{2+} ions on production of acetoin and butane-2:3-diol from a mixture of pyruvic acid and acetaldehyde*

Enzyme (2 ml., 2.02 mg. of nitrogen), 20 μ moles of lithium pyruvate, 100 μ moles of acetaldehyde, 100 μ moles of phosphate buffer, pH 6.2, and varying amounts of magnesium chloride and manganous chloride incubated in air at 37°.

Mg^{2+} (μ moles)	Mn^{2+} (μ moles)	Pyruvate utilized (μ moles/hr./ 2 ml. of enzyme)	Acetoin and butane-2:3-diol produced (μ moles/hr./ 2 ml. of enzyme)
2	—	10.8	14.4
4	—	12.6	16.1
8	—	15.4	18.5
12	—	17.4	21.4
16	—	19.3	22.3
20	—	19.3	24.4
2	2	14.6	20.0
2	4	15.2	20.7
2	10	16.0	21.0
2	20	16.5	20.9

Table 7. *Effect of concomitant production of citrate on formation of acetoin and butane-2:3-diol from pyruvate and acetaldehyde*

Enzyme (2 ml.), 10 μ moles of lithium pyruvate, 100 μ moles of acetaldehyde, 100 μ g. of diphosphothiamine, 2 μ moles of magnesium chloride, 0.2M-potassium phosphate buffer, pH 7.4, in all flasks. Cofactor addition (where indicated): 2 μ moles of manganous chloride, 100 μ g. of DPN, 2400 units of lactic dehydrogenase, 100 μ g. of coenzyme A, 1.2 mg. of cysteine hydrochloride; 30 μ moles of oxaloacetic acid, where added. Incubated in nitrogen at 37°.

Oxalo- acetate	Cofactors	Acetoin and butane-2:3-diol produced (μ moles/mg. of nitrogen/hr.)	Citrate produced (μ moles/mg. of nitrogen/hr.)
—	—	5.6	0
+	—	9.1	0
+	+	11.7	4.2

Table 8. *Effect of thioctic acid on production of citrate, acetoin and butane-2:3-diol from pyruvate*

Reaction mixture: enzyme (2 ml.), 50 μ moles of lithium pyruvate, 30 μ moles of oxaloacetic acid, 100 μ g. of diphosphothiamine, 2 μ moles of magnesium chloride, 0.2M-potassium phosphate buffer, pH 7.4; cofactor additions as in Table 8. Thioctic acid (100 μ g.) was added where indicated. (A solution of thioctic acid was prepared by dissolving 5 mg. in 0.5 ml. of acetone and 4.5 ml. of 0.2M-potassium phosphate buffer, pH 7.4.) Incubated in nitrogen at 37°.

Thioctic acid added (μ g.)	Amounts produced (μ moles/mg. of nitrogen/hr.)		
	Acetoin and butane-2:3-diol	Citrate	Acetate
—	1.35	2.85	0
100	1.50	4.60	0.45

Acetaldehyde in these circumstances depressed the formation of citrate and markedly stimulated production of acetoin and butane-2:3-diol.

DISCUSSION

The main differences between our enzyme preparation and that of Korkes *et al.* (1951) are summarized in Table 10. Both preparations were able to produce acetoin and citrate under appropriate conditions. Further attempts at fractionation of the system by precipitation with ammonium sulphate, or at different pH values, did not produce an enzyme fraction which functioned in the formation only of acetoin and not of citrate. The conclusion, therefore, is that the same enzyme system is concerned in the decarboxylation of pyruvate for the formation of both citrate and acetoin. The different cofactor requirements are produced by different methods of preparation, and it is apparent that our preparation is less pure as it contains condensing enzyme. The observation that the pH optimum for formation of acetoin with pyruvate as sole substrate is different from that for production of citrate does not necessarily indicate that two different enzymes exist. In the presence of acetaldehyde variation in pH from 6.2 to 7.4 did not result in a smaller production of acetoin and butane-2:3-diol, whereas in its absence a decrease occurred. It is therefore possible that the lower pH is concerned with the dissociation of the

acetaldehyde-diphosphothiamine complex to give free acetaldehyde.

It is apparent that the main operative mechanism for production of acetoin in mammalian systems when acetaldehyde is present in excess is the condensation of free acetaldehyde with acetaldehyde-diphosphothiamine complex. If, however, pyruvate is present in excess with a limited amount of acetaldehyde, the main mechanism of production is different. The figures for production of carbon dioxide in this case appear to support the view that some compound which would react as acetoin in our method of estimation is produced without decarboxylation of pyruvate. Juni & Heym (1956) have demonstrated the formation of α -acetyl-lactic acid by mammalian systems. In our system, in the presence of excess of pyruvate, it seems likely that a similar reaction occurs. α -Acetyl-lactate would react as acetoin in our method of estimation, but would not spontaneously undergo decarboxylation at pH 6.2 in the reaction vessel. Thus pyruvic acid can act as an acetaldehyde acceptor leading to the formation of α -acetyl-lactic acid. This does not occur to a significant extent if pyruvic acid is the sole substrate, but does in the presence of an excess of pyruvic acid relative to acetaldehyde. This is probably due to the formation from added acetaldehyde of an acetaldehyde-diphosphothiamine complex, which then uses pyruvic acid as an acetaldehyde acceptor. Oxaloacetate can also be utilized instead of pyruvate as a substrate for formation of acetoin. It will not, however, act as an acetaldehyde acceptor in the same way as pyruvate; thus the only mechanism operating is the double decarboxylation of the oxaloacetate to give acetaldehyde-diphosphothiamine complex which can then condense with free acetaldehyde to yield acetoin.

Attempts to remove any free acetaldehyde produced in the reaction by the use of dimedone as a trapping agent failed. Dimedone exerted a stimulating effect on production of acetoin from pyruvate and from pyruvate + acetaldehyde. This may be a non-specific effect and the dimedone may not have condensed with the acetaldehyde, or the dimedone

Table 9. *Effect of acetaldehyde in presence of thioctic acid on citrate and production of acetoin and butane-2:3-diol from pyruvate*

Reaction mixture was the same as for Table 8. Thioctic acid was added to all flasks; 100 μ moles of acetaldehyde were added where indicated. Incubation was in nitrogen at 37°.

Acetaldehyde	Amounts produced (μ moles/mg. of nitrogen/hr.)		
	Acetoin and butane-2:3-diol	Citrate	Acetate
–	1.15	1.35	0
+	7.85	0.75	0

Table 10. *Differences in preparation and composition between the enzyme preparation of Korkes et al. (1951) (A) and the preparation used in this study (B)*

	A	B
Starting material	Fresh pig-heart muscle	Pig-heart muscle after freezing and thawing
Dispersion medium	0.2M-K ₂ HPO ₄	0.02M-K ₂ HPO ₄
Saturation of ammonium sulphate (%)	0.40 or 0.50	0.30
Added cofactor requirements for production of citrate	Coenzyme A	Thioctic acid
Further purification	Ethanol fractionation	None
Additional enzyme requirements for synthesis of citrate	Condensing enzyme	None

may have trapped the acetaldehyde and in so doing activated it. Thus the condensation of acetaldehyde-diphosphothiamine complex and acetaldehyde-dimedone complex would readily occur. In this way the acetaldehyde-dimedone complex may be a better acetaldehyde acceptor than is free acetaldehyde for condensation to acetoin.

The effects of metal ions on the formation of acetoin can be divided into two groups. The effects of Na^+ and K^+ ions on the system were seen only with pyruvate as sole substrate and at a pH far removed from the optimum. This probably indicates that at pH 6.2 the concentration of H^+ ion is the main factor in securing a dissociation of the acetaldehyde-diphosphothiamine complex, whereas, at a pH where this dissociation is diminished, increased concentrations of K^+ ion will produce a similar, but smaller, effect. The effect of Mg^{2+} and Mn^{2+} ions is not maximum when pyruvate is the sole substrate. Although Mg^{2+} ions are essential for the reaction, the optimum concentration is low. If acetaldehyde was present in addition to pyruvate, the optimum concentration of Mg^{2+} ions was much higher. Magnesium ions are therefore essential not only for the decarboxylation of the pyruvate but also for the condensation reaction with free acetaldehyde. Mn^{2+} ions in very much smaller concentration produced the same effect. In addition Mn^{2+} ions have a greater effect on the mechanism of the production of acetoin, which does not involve pyruvic acid. It is suggested that this mechanism is the condensation of acetaldehyde to yield acetoin. This is not a major mechanism of formation of acetoin but in the presence of 100 μ moles of acetaldehyde and 2 μ moles of Mn^{2+} ions it will account for 25% of the total production of acetoin.

In the investigation of the competitive nature of acetoin and citrate production from a complete system containing CoA, DPN, lactic dehydrogenase, condensing enzyme, cysteine, Mg^{2+} and Mn^{2+} ions, oxaloacetate and pyruvate as substrate, production of citrate was observed and the production of acetoin was greater than that found in the absence of added cofactors. This was due partly to the presence of oxaloacetate, which increased the effective concentration of pyruvate, and partly to the presence of Mn^{2+} ions. The addition of thioctic acid, as expected, stimulated production of citrate without affecting that of acetoin. In these circumstances there was no competition between the production of citrate and of acetoin. If, however, in the presence of optimum amounts of thioctic acid, acetaldehyde is added as an additional substrate, there is a diminution in the amount of citrate formed and a marked stimulation of acetoin formation. This not only changes the route of pyruvate dissimilation but also increases the total amount of pyruvate utilized. In a system not producing citrate Juni

(1952) found that acetaldehyde would increase utilization of pyruvate. Acetaldehyde can therefore compete with thioctic acid for the products of decarboxylation of pyruvate, and in so doing it markedly accelerates the rate at which pyruvate is metabolized.

The main conclusions are therefore that four routes of formation of acetoin exist: (1) With pyruvate as sole substrate, condensation of acetaldehyde-diphosphothiamine complex with acetaldehyde liberated from the complex. (2) Condensation of acetaldehyde-diphosphothiamine complex derived from pyruvate with added acetaldehyde. (3) Condensation of acetaldehyde-diphosphothiamine complex with pyruvate to give α -acetolactic acid. (4) Condensation of acetaldehyde-diphosphothiamine complex derived from acetaldehyde in the presence of Mn^{2+} ions with free acetaldehyde.

Of these four routes (2) seems to be the most significant and it can act as a direct competitor with the pathway leading via acetyl lipoate, acetyl-CoA and condensation with oxaloacetate to form citrate.

The significance of these observations for variations in the level of acetoin in human blood depends largely on the significance of acetaldehyde metabolism in man. Although the blood level of free acetaldehyde is about 50–100 $\mu\text{g./100 ml.}$, the total amount of acetaldehyde which can be extracted from blood, mainly from acetaldehyde-protein complexes, is about 90 mg./100 ml. (Barker, 1941; Stotz, 1943). The problem of the metabolic reactivity of this large amount of acetaldehyde present in blood remains.

SUMMARY

1. Pyruvate and mixtures of pyruvate and acetaldehyde will both act as substrates for formation of 3-hydroxybutan-2-one by the use of a pig-heart preparation capable of formation of citrate.
2. The formation of α -acetyl-lactic acid by the system has been indicated in the presence of a large excess of pyruvic acid relative to acetaldehyde.
3. Manganese (Mn^{2+}) ions stimulate the production of 3-hydroxybutan-4-one from acetaldehyde.
4. Acetaldehyde and thioctic acid compete for the products of decarboxylation of pyruvic acid by this system.

We thank Professor Frank C. Happold for his helpful advice and criticism and also the Medical Research Council for a grant supporting one of us (M. W.).

REFERENCES

- Barker, S. B. (1941). *J. biol. Chem.* **137**, 783.
Berg, R. L. & Westerfeld, W. W. (1944). *J. biol. Chem.* **152**, 113.
Dixon, M. (1934). *Manometric Methods*. Cambridge University Press.

- Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
- Happold, F. C. & Spencer, C. P. (1952). *Biochim. biophys. Acta*, **8**, 18.
- Juni, E. (1952). *J. biol. Chem.* **195**, 727.
- Juni, E. & Heym, G. A. (1956). *J. biol. Chem.* **218**, 365.
- Korkes, S., del Campillo, A., Gunsalus, I. C. & Ochoa, S. (1951). *J. biol. Chem.* **193**, 721.
- Krampitz, L. O. (1948). *Arch. Biochem.* **17**, 81.
- Langenbeck, W. & Faust, G. (1953). *Hoppe-Seyl. Z.* **292**, 73.
- McLendon, J. F. (1944). *J. biol. Chem.* **154**, 357.
- Markham, R. (1942). *Biochem. J.* **36**, 790.
- Ochoa, S., Stern, J. R. & Schneider, M. C. (1951). *J. biol. Chem.* **193**, 691.
- Schweet, R. S. & Cheslock, K. (1952). *J. biol. Chem.* **199**, 749.
- Singer, T. P. & Pensky, J. (1952). *Biochim. biophys. Acta*, **9**, 316.
- Slade, H. D. & Werkman, C. H. (1943). *Arch. Biochem.* **2**, 105.
- Stern, J. R., Shapiro, B., Stadtman, E. R. & Ochoa, S. (1951). *J. biol. Chem.* **193**, 703.
- Stotz, E. (1943). *J. biol. Chem.* **148**, 585.
- Stotz, E. (1954). *J. biol. Chem.* **208**, 591.
- Strecker, H. J. & Ochoa, S. (1954). *J. biol. Chem.* **209**, 313.
- Taylor, T. G. (1953). *Biochem. J.* **54**, 48.
- Weil-Malherbe, H. (1937). *Biochem. J.* **31**, 2202.
- Wendel, W. B. (1931). *J. biol. Chem.* **94**, 717.

β -Glucuronidase Activity During the Early Development of *Xenopus laevis* Embryos

By F. BILLETT

Institute of Animal Genetics, University of Edinburgh

(Received 21 February 1957)

β -Glucuronidase has been associated with the proliferation of cells in normal and neoplastic tissue (Levy, Kerr & Campbell, 1948; Fishman & Anylan, 1947). Rapid cell divisions are characteristic of certain phases of embryonic development; it is therefore of some interest to study the behaviour of β -glucuronidase during the early development of embryos. The possible implication of steroids in the primary differentiation of the amphibian embryo (Waddington, Needham, Nowinski, Needham & Lemberg, 1934) adds interest to such a study, particularly in view of the suggestion of Fishman (1947) that β -glucuronidase may be connected with the metabolism of steroids in mammalian tissues. Apart from these special considerations, there is clearly a general need for more information on the behaviour of enzymes during early embryonic development.

The South African clawed toad (*Xenopus laevis*) was chosen for this investigation because large numbers of fertile eggs can be obtained from these animals, at any time of the year, by injections of chorionic gonadotrophin. Initially a number of determinations showed that a small amount of β -glucuronidase activity was present in extracts of embryos, ranging from two-cell to newly hatched larvae. In this paper the results of a large number of determinations show that small, real variations of activity occur during the early stages of development. A preliminary account of this work has been given elsewhere (Billett, 1956).

EXPERIMENTAL

Fertilized eggs were obtained from the toads by injection of chorionic gonadotrophin. Egg capsules were removed by papain and thioglycollate (Spiegel, 1951). Approximately 300 embryos of about the same stage were selected and divided into groups of about 60. After several washes in Holtfreter solution diluted ten times with water (Hamburger, 1947) the embryos were allowed to develop, in the solution, at 18°. The development of the embryos was stopped at a known time by placing them in a deep-freeze at -20°. Before freezing the embryos were grouped in stages according to the second column of Table 1. This staging is based on a description of the external appearance of the embryos at intervals of approx. 2 hr. at 18°. The stages chosen correspond fairly well with those of Nieuwkoop & Faber (1956).

After 2-14 days at -20°, each sample was homogenized at about 5° in a small quantity of water and made up to 1 or 2 ml. An all-glass homogenizer of the Potter-Elvehjem type was used, operated by hand. The material was readily broken down during the course of 1 min. This operation was carried out at about 5°. Homogenates were stored in a refrigerator overnight and then centrifuged at about 3000 g. The centrifuging resulted in a fractionation similar to that described by Panijel (1950). Residues were washed once by centrifuging in the original volume of water. The original supernatant, together with the washing from the residue, constituted the enzyme extract. Microscopic examination of the residue showed it to consist largely of intact yolk platelets.

The β -glucuronidase activity of the extracts was determined by a method previously described (Billett, 1954). This method is based on the colorimetric determination of 8-hydroxyquinoline formed by the hydrolysis of its