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Metabolism of Acetoin in Mammalian Liver Slices and Extracts

INTERCONVERSION WITH BUTANE-2,3-DIOL AND BIACETYL

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1. [^{14}C]Acetoin was enzymically synthesized from [^{14}C]pyruvate with a pyruvate decarboxylase preparation. Its optical activity was $[\alpha]_D^{20}$ −78°. 2. Large amounts (1000-fold higher than physiological concentrations) of acetoin were incubated with rat liver mince. Acetoin disappeared but very little $^{14}CO_2$ was evolved. A compound accumulated, which was purified and identified as butane-2,3-diol. Chromatography on borate-impregnated paper indicated the presence of both the *erythro* and *threo* forms. 3. Liver extracts capable of interconverting biacetyl, acetoin and butane-2,3-diol were obtained. These interconversions were catalysed by two different enzymes: acetoin dehydrogenase (EC 1.1.1.5) and butane-2,3-diol dehydrogenase (EC 1.1.1.4), previously identified in bacteria. Both required NAD^+ or $NADP^+$ as cofactors and were different from alcohol dehydrogenase. The equilibrium in both cases favoured the more reduced compound. 4. The activity of butane-2,3-diol dehydrogenase was decreased by dialysis against EDTA: the addition of Co^{2+}, Cu^{2+}, Zn^{2+} and other bivalent metal ions restored activity. 5. Biacetyl reductase was resolved into multiple forms by CM-Sephadex chromatography and electrophoresis.

Acetoin (3-hydroxybutan-2-one) is produced in significant amounts as a by-product of the mammalian pyruvate decarboxylase reaction (EC 4.1.1.1) (Schweet, Fuld, Cheslock & Paul, 1951). This production is increased by the acetaldehyde formed after the consumption of alcohol (Lubin & Westerfeld, 1945). Acetoin also occurs as a minor constituent of many foodstuffs.

The catabolism of acetoin in mammals has received little attention. Dogs (Greenberg, 1943) and cats (Dawson & Hullin, 1954*a*,*b*) injected with the compound metabolized it rather rapidly.

Järnefelt (1953, 1955) reported that liver slices, but not homogenates, were capable of converting acetoin into butane-2,3-diol; however, the product was never isolated and was identified solely on the basis of acetaldehyde production in the presence of periodate.

In the present paper we report the enzymic synthesis and purification of [^{14}C]acetoin and the isolation, identification and determination of the isomeric form of the butane-2,3-diol produced on incubation of acetoin with rat liver mince. We also report the preparation of cell-free extracts from mammalian liver capable of reducing acetoin and biacetyl, and the partial purification and some of the properties of the enzymes involved. Our results indicate that in mammalian liver, separate enzymes catalyse the reduction of acetoin and biacetyl.

MATERIALS AND METHODS

Preparation of liver mince. Albino rats of the Sprague–Dawley strain and rabbits were obtained from the animal house of the American University of Beirut. Animals were killed by decapitation and the liver was quickly removed. A mince was prepared by cutting the livers into small pieces (less than 2mm across) with scissors.

Preparation of acetone-dried powder extracts. Rat or rabbit livers (freed from gall bladder) were blended for 1min with 10vol. of acetone at −18°C and quickly filtered by suction. The cake was reblended twice with the same volume of acetone. Finally it was dried over P_2O_5 *in vacuo*; storage was in screw-cap bottles over $CaSO_4$ at −12°C.

Extracts were prepared by homogenizing the powder for 2min in a Potter–Elvehjem homogenizer at 0–4°C with 5vol. of 0.05M-sodium phosphate buffer, pH7.4, and centrifuging at 8000*g* for 15min.

Fresh liver extract. Fresh liver was cut into small pieces and homogenized in a Potter–Elvehjem homogenizer at 0°C with 5vol. of 0.05M-sodium phosphate buffer, pH7.4, and centrifuged at 9000*g* for 15min.

Chemicals. [^{14}C]Pyruvate was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Acetoin, obtained from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.), was twice distilled at 50°C and 2–5mmHg. The distillate was left to dimerize at 4°C and the yellowish-white solid obtained was washed with alcohol and dried *in vacuo*. Butane-2,3-diol obtained

from K & K Laboratories (Plainview, N.Y., U.S.A.) was distilled at 70–80°C and 2–5mmHg, the first and last fractions being discarded.

Biacetyl (purissima) was obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.) and used without further purification.

Radioactivity measurements. Samples were either dried on concentric planchets and counted for radioactivity in a gas-flow counter with an efficiency of 30% and corrected for self-absorption whenever necessary or added to a sample of a solution of 4g of 2,5-diphenyloxazole and 50mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene and the radioactivity was counted in a liquid-scintillation spectrometer and corrected for efficiency by the internal-standard method. For comparison radioactivity is expressed as d.p.m.

Determination of acetoin. Acetoin was determined by the method of Westerfeld (1945). The method was modified for solutions that had been treated with $HClO_4$ to remove protein, then neutralized with KOH by passing the neutral protein-free filtrate [3ml of sample+1ml of 10% (w/v) sodium tungstate+1ml of 0.33M-H_2SO_4] over a mixed-bed [Dowex 1 (Cl^- form)+Dowex 50 (H^+ form)] column (0.2cm×3cm), washing with water and collecting the first 10ml of eluate. A portion (5ml) was taken and subjected to the procedure of Westerfeld (1945).

Detection of butane-2,3-diol on paper. When samples were subjected to ascending chromatography on Whatman 3MM paper with ethyl acetate–ethanol–water (12:2:1, by vol.) as the solvent system (Hais & Macek, 1963*a*) butane-2,3-diol was detected by the periodate–benzidine spray (Hais & Macek, 1963*b*). However, after chromatography on paper impregnated with borate the dried paper was sprayed lightly with water, suspended for 1h in a jar containing acetic acid, aerated for 10min and finally the material detected by the periodate–benzidine method.

Preparation of [^{14}C]*acetoin.* [2,3-^{14}C]Acetoin was prepared by incubating 15μmol of [2-^{14}C]pyruvate and unlabelled acetaldehyde with 5ml of pyruvate decarboxylase prepared from ox heart (Green, Westerfeld, Venesland & Knox, 1942) in 0.05M-glycerophosphate buffer, pH6.0, containing 0.5mg of $MgSO_4$ and 0.1mg of thiamin pyrophosphate. The enzyme (40–50mg of protein/ml) was assayed and found to form at least 3μmol of acetoin/h per ml at saturation with the substrates. Preliminary trials showed that an acetaldehyde/pyruvate molar ratio of 6:1 produced the highest yield of radioactivity in acetoin (up to 60% of the added pyruvate).

A neutralized protein-free filtrate of the incubation mixture was distilled and the distillate passed over a mixed-bed column and distilled again in the presence of dimedone to trap acetaldehyde. The distillate obtained was chromatographed on a column (2cm×50cm) of strongly basic resin [Amberlite IRA-400 (bisulphite form)] prepared as described by Gabrielson & Samuelson (1952) and eluted with water followed by 0.5M-NaCl (adjusted to pH7.0).

The pooled eluates in the acetoin peak were concentrated by vacuum distillation in the presence of sodium bisulphite, which complexes the acetoin, followed by the disruption of the acetoin–bisulphite complex with solid Na_2CO_3 and distillation. The purified aqueous acetoin solution was stored at −12°C. Labelled biacetyl was prepared by oxidizing a sample of [^{14}C]acetoin with the $FeCl_3$–$FeSO_4$–H_2SO_4 mixture of Westerfeld (1945). The biacetyl was distilled *in vacuo* and collected at −50°C.

Identity and radioactive purity of the biosynthesized acetoin. When [2-^{14}C]pyruvate with a specific radioactivity of 31mCi/mmol was used in the original incubation mixture the purified [2,3-^{14}C]acetoin obtained had a specific radioactivity of 27.1mCi/mmol. The overall radiochemical yield was approx. 20%.

The radioactive purity of the prepared acetoin was checked by its migration as a single peak on Celite-column chromatography in the system of Neish (1950) and on gel-filtration chromatography through a Sephadex G-10 (Pharmacia, Uppsala, Sweden) column. Carrier acetoin was added to a sample and the 2,4-dinitrophenyl-osazone and the -semicarbazone derivatives were prepared. Both were recrystallized to constant specific radioactivity. A sample of the [2,3-^{14}C]acetoin was incubated for 24h with $NaIO_4$ at pH4.5 in a tube through which N_2 was passed gently into a trap containing 2,4-dinitrophenylhydrazine. The acetaldehyde (from C-3 and C-4 of acetoin) was collected as the hydrazone in the trap and counted for radioactivity. The acetic acid formed (from C-1 and C-2) was counted for radioactivity in the original solution. The ratio of ^{14}C in C-2 and C-3 of acetoin was 6:1.

A similar preparation with [3-^{14}C]pyruvate gave [1,4-^{14}C]acetoin in a similar yield. A large-scale incubation of non-radioactive pyruvate and acetaldehyde was done with the same enzyme preparation and essentially the same purification procedure was used as for the preparation of the radioactive acetoin. The optical rotation of the produced acetoin was $[\alpha]_D^{20}$ −78°, indicating that it was essentially the D(−) isomer (reported values, $[\alpha]_D^{20}$ −70° to −79°; Tanko, Munk & Abonyi, 1940). We found this a more convenient method of synthesis than the chemical method of Brady, Rabinowitz, Van Baalen & Gurin (1951); in addition it yields the D(−)-isomer rather than the racemic mixture.

Enzyme assays. All enzyme assays were found to be proportional to enzyme concentration over the range of 0.005–0.04 E_{340} units/min.

Biacetyl reductase and acetoin reductase activities were assayed by measuring in a recording spectrophotometer (Bausch and Lomb 505) the decrease in E_{340} of a mixture of 0.1ml of 1M-acetoin or 1M-biacetyl, 0.1ml of NADH (1.4mg/ml) (Sigma Chemical Co., St Louis, Mo., U.S.A.) and 0.7ml of 0.5M-sodium phosphate buffer, pH6.0, in a 1cm-light-path 1ml quartz cuvette. The reaction was started by adding 0.1ml of the enzyme solution to both the reaction cuvette and the blank cuvette. In the blank cuvette the substrate solution was replaced by water. The blank cuvette was placed in the sample holder and the reaction cuvette in the blank holder. With higher concentrations of NADH it was necessary to open the slit of the spectrophotometer to admit more light.

The same reactions were assayed in the reverse direction by measuring the increase in E_{340} of a mixture containing either 0.1ml of 1M-acetoin or 0.1ml of 30% (v/v) butane-2,3-diol, 0.1ml of semicarbazide solution (25mg/ml) (only with butane-2,3-diol dehydrogenase), 0.1ml of NAD^+ (20mg/ml) and 0.5M-sodium pyrophosphate buffer, pH9.0, to make up to 0.9ml. The reaction was started by

the addition of 0.1 ml of the enzyme solution. The blank contained all the constituents except the substrate.

Alcohol dehydrogenase (EC 1.1.1.1) was also assayed by the same procedure, 0.1 ml of 30% (v/v) propan-1-ol being used as substrate.

Protein determination. Protein was determined by the biuret method of Gornall, Bardawill & David (1949) with crystalline bovine albumin as standard. For dilute protein solutions the method of Warburg & Christian (1941) was used.

Chromatographic separation. Acetoin, butane-2,3-diol and biacetyl were separated by partition chromatography on Celite columns by using water as the stationary phase and ethyl acetate saturated with water as the mobile phase (Neish, 1950). A portion (45 g) of Celite 545 (Koch–Light Laboratories Ltd.) was triturated with 20 ml of water in a mortar, then packed in a column by adding the powder in small portions and packing with a thick glass rod. The sample was triturated with double its weight of Celite and packed on top and the column was developed with the mobile phase.

RESULTS

Accumulation of butane-2,3-diol. Liver mince (2 g) was incubated at 37°C for 2 h with 22.7 μmol of [2,3-^{14}C]acetoin (1.3×10^6 d.p.m.) and 4 ml of Krebs–Ringer phosphate buffer (Krebs & Henseleit, 1932) in a flask provided with a well containing sodium hydroxide to trap the CO_2. The incubation was terminated by the addition of perchloric acid to a final concentration of 0.3 M. From acetoin determinations in a large number of such incubations it was noted that about 70% of the added acetoin (colour reaction) disappeared. Less than 0.5% of the radioactivity appeared in CO_2. These results indicate the accumulation of a metabolite.

Isolation of butane-2,3-diol. The protein-free filtrate obtained from the perchloric acid treatment was cooled to 0°C and neutralized with potassium hydroxide. The precipitated potassium perchlorate was centrifuged off and the neutral supernatant passed through a strongly basic resin [Dowex 1 (X8; carbonate form)] column and washed with water. The clear colourless eluate, containing more than 95% of the radioactivity, was passed through a column (1 cm × 100 cm) of Sephadex G-10 equilibrated and developed with water. Only a single peak could be detected, which moved to the same position as the one obtained with a zero-time control similarly treated. However, when the neutralized Dowex-treated solution was separated by using the same Sephadex G-10 column equilibrated and developed with 0.2 M-sodium bisulphite, two widely separated radioactive peaks were observed (Fig. 1*b*). The minor peak (first peak) containing about 35% of the radioactivity was identified as the acetoin–bisulphite complex by its colour reaction and the increase in its size when a sample of the protein-free filtrate was mixed with radioactive acetoin and the separation repeated on the same column (Fig. 1*a*).

Identification of butane-2,3-diol. A preparative-scale reaction mixture was treated and chromatographed as described above for the isolation of butane-2,3-diol. The fractions of the second peak eluted from the Sephadex G-10 column (4 cm × 100 cm) equilibrated with sodium bisulphite were pooled and authentic butane-2,3-diol was added to the solution. The solution was extracted continuously with peroxide-free ether and the ether was evaporated to give a viscous yellow liquid. The

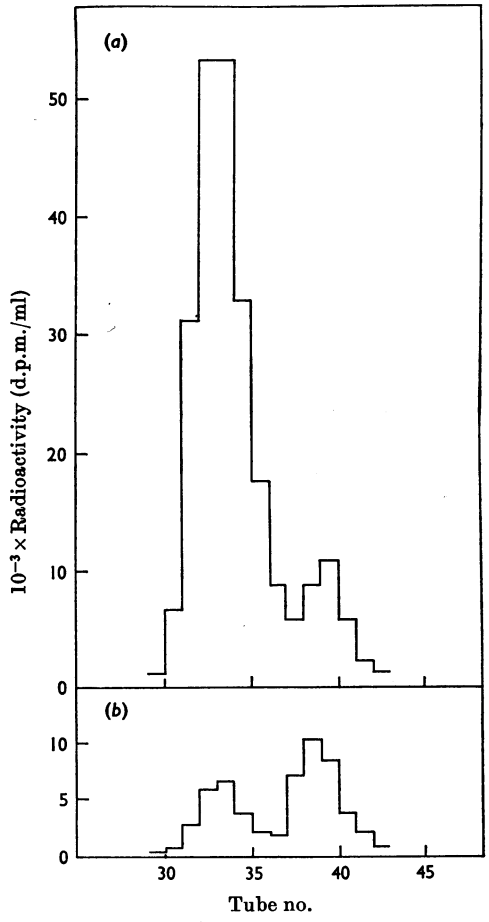

Fig. 1. Chromatography of incubation mixture on Sephadex G-10 equilibrated with bisulphite. The Sephadex G-10 column (1 cm × 110 cm) was equilibrated and developed with 0.2 M-sodium bisulphite, at a flow rate of 20 ml/h. Fractions (2 ml) were collected. In (*b*) the 1 ml of incubation mixture was separated; in (*a*) 1 ml of the same incubation mixture was mixed with 0.1 ml of [2,3-^{14}C]-acetoin (2.7×10^5 d.p.m.) and separated as described in the text.

liquid was treated with saturated 2,4-dinitrophenylhydrazine in 0.5M-sulphuric acid, followed by addition of activated charcoal. The preparation was filtered and the clear solution obtained was found to contain more than 90% of the radioactivity of the second peak.

Chromatography of the solution on Whatman 3MM paper with ethyl acetate–ethanol–water (Hais & Macek, 1963*a*) revealed a single coloured spot on development of the chromatogram with the periodate–benzidine method (Hais & Macek, 1963*b*). This spot contained all the radioactivity and also corresponded to the spot obtained with authentic butane-2,3-diol.

A sample of the solution containing 4200d.p.m. consumed 0.20mmol of periodate (Birkinshaw, Charles & Clutterbuck, 1931). The solution was gassed with N_2 into a 2,4-dinitrophenylhydrazine trap, and the hydrazone derivative, crystallized three times from hot ethanol, melted at 167°C (m.p. of acetaldehyde hydrazone 167°C). Its specific radioactivity was 35.5d.p.m./mg, corresponding to the calculated 36d.p.m./mg if all the radioactivity was in butane-2,3-diol, and was quantitatively cleaved with periodate to 2mol of acetaldehyde/mol of butane-2,3-diol.

A phenylurethane derivative of the metabolite was prepared and precipitated from hot carbon tetrachloride to a constant specific radioactivity of 11 and 11.2c.p.m./mg on the second and third precipitations. The derivative did not form uniform crystals.

When the metabolite was chromatographed on borate-impregnated Whatman 3MM paper with ethyl acetate–ethanol–water two radioactive spots with R_F values of 0.81 and 0.55 were detected. About two-thirds of the radioactivity was distributed in the faster-moving spot and one-third in the slower-moving spot (Fig. 2).

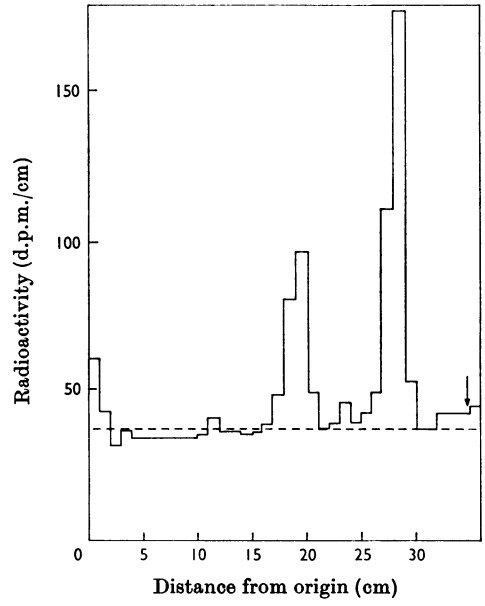

Fig. 2. Ascending chromatography of the metabolite on sodium borate-impregnated Whatman 3MM paper, with ethyl acetate–ethanol–water (12:2:1, by vol.) as solvent. The paper was cut into 1 cm strips and their radioactivities measured in a liquid-scintillation counter. ----, Background counts. The arrow indicates the solvent front.

Properties of extracts. Liver extracts by themselves did not utilize acetoin. However, the addition of NAD^+, NADH, $NADP^+$ or NADPH to crude cell-free extracts of fresh liver restored activity. These supplemented extracts took up either acetoin or biacetyl (colour reaction of Westerfeld, 1945) at a rate equal to that observed with the equivalent amount of liver mince. The activity with NAD^+ was twice that with $NADP^+$. The acetone-dried powder extract was also found to be active on addition of NAD^+ or $NADP^+$.

Partial purification of biacetyl reductase. Biacetyl reductase was partially purified from rat liver; Table 1 summarizes the procedure. An eightfold purification of biacetyl reductase was obtained with a yield of 35%; some activation occurred at each dialysis step.

The same procedure caused less than twofold purification of acetoin reductase, with a yield of 7%. Similar results were obtained with rabbit liver.

When the purified preparation was applied to a column (5cm×55cm) of CM Sephadex-50 equilibrated with 0.01M-sodium phosphate buffer, pH 6.7, and eluted with the same buffer, the complex profile shown in Fig. 3 was obtained. Biacetyl reductase activity appeared in at least two peaks, none of which was identical with the acetoin reductase peak. Rechromatography of the second large biacetyl reductase fraction on the same column led to a similar separation of the biacetyl reductase into two peaks in about the same positions.

Effect of ethanol on enzyme activities. An acetone-dried powder extract of rat liver was divided into three samples. To two samples ethanol was added slowly to a final concentration of 10 and 20% (v/v) respectively, and the solutions were left at 0°C for 10min and centrifuged at 3000*g* for 15min. The third sample was not treated. All three supernatants were adjusted to 60% saturation with ammonium sulphate; the precipitates were dissolved in sodium phosphate buffer and the activities of alcohol dehydrogenase, butane-2,3-diol dehydrogenase and acetoin dehydrogenase tested. In the untreated crude extract the proportions of the acti-

Table 1. *Purification of biacetyl reductase and acetoin reductase from fresh rat liver*

The ammonium sulphate fraction was dialysed overnight against 5mM-sodium phosphate buffer, pH6.5, and applied to a column (2cm×25cm) of DEAE-cellulose equilibrated with 5mM-sodium phosphate buffer, pH6.5, and eluted with the same buffer. The second dialysate refers to the eluate from the DEAE-cellulose, which was concentrated by precipitation with $(NH_4)_2SO_4$ at 66% saturation and dissolved in 0.01M-sodium phosphate buffer, pH6.7, and dialysed overnight against the same buffer.

			Biacetyl reductase			Acetoin reductase			Biacetyl reductase/ acetoin reductase activity ratio
Preparation	Volume (ml)	Protein (mg/ml)	Specific activity (nmol/min per mg of protein)	Total activity (μmol/min)	Yield (%)	Specific activity (nmol/min per mg of protein)	Total activity (μmol/min)	Yield (%)	
Homogenate	110	55	169	1020	100	3.7	23	100	46
40–60%-satd. $(NH_4)_2SO_4$	31	41	556	702	68	5.8	7.4	32	95
Dialysate	32	27	887	765	75	5.8	5.0	22	154
After DEAE-cellulose chromatography	60	4	1330	320	31	7.4	1.8	8	180
Dialysate	26	10	1370	340	35	6.4	1.6	7	213

vities were 1:2.7:4.7 respectively. Exposure of the enzyme preparation to 10% (v/v) ethanol destroyed the alcohol dehydrogenase activity completely, but the other two dehydrogenases were not affected significantly.

Product identification. [^{14}C]Biacetyl was incubated with NADH and an acetone-dried powder extract of rat liver for 4h. The product was separated on a Celite column. A double peak representing more than 50% of the radioactivity appeared in the acetoin and butane-2,3-diol regions (Fig. 4). This peak could not be detected in the zero-time control.

The reversal of the acetoin dehydrogenase reaction could not be demonstrated unless an NADH acceptor was included. When [^{14}C]acetoin was incubated with NAD^+, phenazine methosulphate, 1mg of Nitro Blue Tetrazolium and an acetone-dried powder extract under the conditions specified in Fig. 5, and the product fractionated on the Celite column, a small peak representing 1.4% of the total radioactivity (c.p.m.) was detected in the biacetyl region. In a control without enzyme the biacetyl region contained only 0.24% of the radioactivity.

Metal requirement for butane-2,3-diol dehydrogenase. Dialysis of the enzyme preparation against 1mM-EDTA in 0.01M-sodium phosphate buffer, pH7.4, followed by dialysis against the phosphate buffer decreased the enzyme activity to one-third of that of a control enzyme preparation dialysed only against the phosphate buffer. The enzyme could be reactivated to 90% of its initial activity by adding Co^{2+} to a final concentration of 0.1mM. Cu^{2+} and Zn^{2+} were slightly less effective at the optimum concentration of 0.1mM, followed by Mn^{2+}, Fe^{2+}, Mg^{2+} and Ca^{2+}.

Effect of high NADH concentration. A sharp decrease in the activity of acetoin reductase occurred when the NADH concentration was increased; at 2mM-NADH the activity was less than 2% of the activity obtained at 0.2mM.

pH *optimum and apparent* K_m. The pH optimum for both reductase activities was near 6.0. At 0.2mM-NADH and pH6.0 the apparent K_m of acetoin reductase was calculated from a Lineweaver–Burk plot to be 50mM for acetoin. The apparent K_m for biacetyl reductase under the same conditions was 48mM-biacetyl.

Electrophoresis of acetoin dehydrogenase. An acetone-dried powder extract of rat liver was subjected to electrophoresis at pH8.6 on cellulose acetate strips (Millipore Corp., Bedford, Mass., U.S.A.) at 20V/cm for 1h. The position of the biacetyl reductase was located by the application of a second strip moistened with a mixture of 1ml of 1M-acetoin, 0.3ml of phenazine methosulphate (1mg/ml), 1ml of water, 10mg of NAD^+ and 3ml of

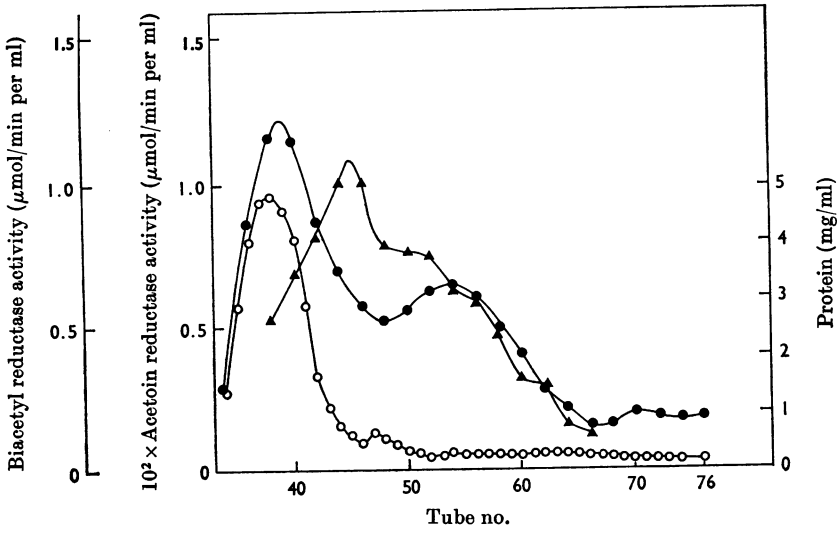

Fig. 3. Separation of acetoin reductase and biacetyl reductase on CM-Sephadex. A portion (10 ml) of the dialysate after DEAE-cellulose treatment (Table 1) was applied to a column (5 cm × 55 cm) of CM-Sephadex equilibrated for several days and developed with 0.01 M-sodium phosphate buffer, pH 6.7, at a flow rate of 150 ml/h. Fractions (8 ml) were collected. ●, Biacetyl reductase; ○, acetoin reductase; ▲, protein.

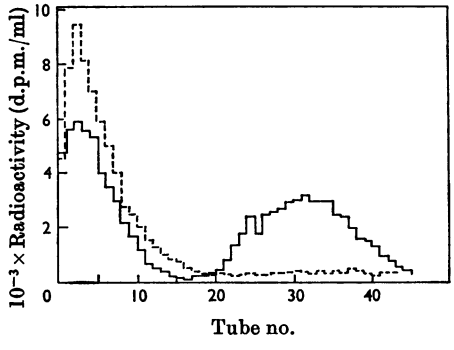

Fig. 4. Celite column chromatography of the incubation mixture of [^{14}C]biacetyl and partially purified biacetyl reductase. [1,4-^{14}C]Biacetyl (4 nmol; 300 000 d.p.m.) was incubated at 37°C for 4 h with 0.4 mg of NADH, 1.5 ml of 0.1 M-sodium phosphate buffer, pH 7.4, and 0.1 ml of the acetone-dried powder extract. The mixture was separated on a column (2 cm diam.) containing 90 g of Celite 545 and developed with ethyl acetate saturated with water. Fractions (3 ml) were collected. The first peak corresponds to the position of biacetyl and the second to that of acetoin and butane-2,3-diol. ——, Experimental; – – – –, control.

Nitro Blue Tetrazolium (1 mg/ml) (Gelman Instrument Co. Manual no. 701 76-A; P.O. Box 1448, Ann Arbor, Mich., U.S.A.). At least two dark bands of precipitated Nitro Blue and a number of minor bands appeared.

Stereospecificity of acetoin reductase. The partially purified acetoin reductase was incubated with NADH and D(−)-acetoin. The butane-2,3-diol formed was chromatographed on borate paper and detected by the modified periodate–benzidine spray (Hais & Macek, 1963*b*). Under these conditions acetoin does not give a spot. Two spots with R_F values of 0.81 and 0.55 were seen.

DISCUSSION

When liver mince was incubated with labelled acetoin at a concentration of 4.5 mM, which is about 1000-fold the physiological blood concentration of acetoin (Dawson & Hullin, 1954*a*,*b*), very little labelled CO_2 was detected and the major portion of the acetoin (about 70%) was reduced to butane-2,3-diol. At lower concentrations of acetoin much more of it is converted into CO_2 (M. A. Gabriel & U. A. S. al-Khalidi, unpublished work).

Butane-2,3-diol cannot be separated from acetoin by ordinary gel filtration. However, when the filtration is performed in the presence of bisulphite, which forms a complex with acetoin, the two compounds are well separated. This technique may find general application in the separation of sugars or other compounds when it is possible to form complexes with bisulphite or other complexing agents.

The butane-2,3-diol, produced from D(−)-[^{14}C]-acetoin by both liver mince and partially purified

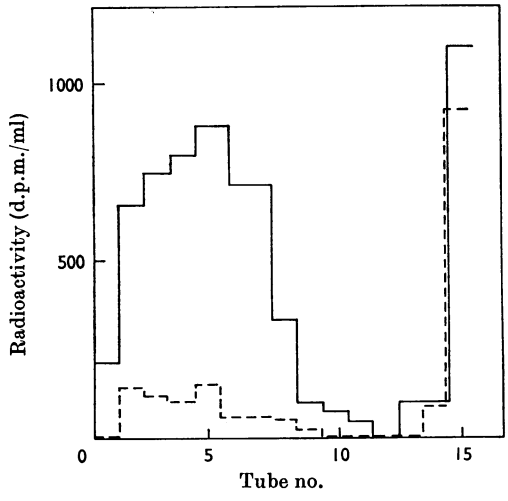

Fig. 5. Celite column chromatography of the incubation mixture of [^{14}C]acetoin with acetoin dehydrogenase. Acetoin (13nmol; 1×10^6 d.p.m.) was incubated in a closed tube for 3h at 37°C with 10mg of NAD^+, 0.5ml of partially purified biacetyl reductase in 0.5M-sodium pyrophosphate buffer, pH9.0, 0.5mg of phenazine methosulphate and 1mg of Nitro Blue Tetrazolium in a total volume of 4.0ml. The mixture was chromatographed on a column (2cm diam.) containing 68g of Celite 545 and developed with ethyl acetate saturated with water. Fractions (3ml) were collected. The first peak coincides with biacetyl. ——, Experimental; ----, control.

acetoin reductase, was separated on borate-impregnated paper into two spots with R_F values of 0.55 and 0.81. It is assumed that the spot with R_F 0.55 represents the *erythro* form (*meso*) reacting with borate to yield a more polar compound with retarded mobility. The other spot would represent the *threo* (D and/or L) form. Quantitatively the *erythro* spot contained about one-third of the radioactivity. As Taylor & Juni (1960) pointed out in their study of butane-2,3-diol dehydrogenase in bacteria, one enzyme and one form of substrate cannot account for the appearance of two forms of butane-2,3-diol. However, the existence of an isomerase or two reductases may yield the two forms.

This is the first time that mammalian enzyme preparations capable of the interconversion of acetoin, biacetyl and butane-2,3-diol have been reported. Juni & Heym (1957) reported that the bacterial acetoin reductase (EC 1.1.1.4) and biacetyl reductase (EC 1.1.1.5) activities reside in one enzyme, in contrast with the report of Strecker & Harary (1954).

We find that in liver extracts different enzymes catalyse the two reactions. Both enzyme activities are distinct from alcohol dehydrogenase, as the product after CM-Sephadex treatment contained very little alcohol dehydrogenase activity, and ethanol treatment resulted in the complete loss of activity of alcohol dehydrogenase.

The biacetyl reductase was resolved on CM-Sephadex into at least two peaks. Two bands were also seen on electrophoresis. The multiple forms of biacetyl reductase may be explained on the basis of isoenzymes or of equilibrium of multiple forms of the same enzyme. In favour of the second possibility is the finding of the same complex profile on re-chromatography of one of the fractions on CM-Sephadex.

The equilibrium of both reactions is in favour of the reduced compound. The reaction catalysed by biacetyl reductase is almost irreversible.

The apparent K_m values obtained for both enzymes are extremely high; the expected physiological concentrations of the substrates are probably two or three orders of magnitude lower than these values. This suggests that the enzymes have very little activity under normal conditions. The possibility that both enzymes are primarily specific for other substrates cannot be ruled out. However, in special cases where acetoin (Dawson & Hullin, 1954*a*,*b*), biacetyl or ethanol (Lubin & Westerfeld, 1945) is consumed the tissue substrate contents may become high enough to make the activity of the enzymes significant.

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