OXIDATIONS IN *ACETOBACTER SUBOXYDANS**

by

TSOO E. KING AND VERNON H. CHELDELIN

Department of Chemistry and the Science Research Institute, Oregon State College, Corvallis, Oregon (U.S.A.)

Previous papers from this laboratory[1] have reported the oxidation of some non-nitrogenous substances in *Acetobacter suboxydans*. Resting cells can oxidize glycerol or sorbitol beyond the dihydroxyacetone (DHA) or sorbose stage. Acetate and other intermediates of the tricarboxylic acid cycle are inert. In this paper, the oxidation of other substances, including some phosphorylated compounds is reported, together with further attempts to show acetate utilization and the confirmation of previous results by use of cell-free enzymes.

Preparation of cell-free extracts

A. suboxydans ATCC 621 cells were grown as previously described[1]. A simplified medium which consisted of 1 % yeast, 5 % glycerol and 0.5 % phosphate buffer at pH 6.0 was also used in some experiments, with no significant differences noted among the cells harvested.

Attempts to prepare cell-free extracts by many previously described methods were unsuccessful. Electron microscopic examination revealed very little rupture of the cell walls after freezing and thawing, sonic vibration with various abrasives in a MICKLE vibrator[2], acetone precipitation[3], "sloppy" drying[4], grinding with powdered glass[5], or grinding in a mill *in vacuo*[6]. However, grinding of lyophilized or fresh cells with alumina gave an extract which could oxidize certain substrates. The preparation was similar to those of MCILWAIN[7] and HAYAISHI AND STANIER[8]. A typical run was as follows: A mixture of 5 g of lyophilized cells and 20 g alumina (Alcoa #301) was mixed well with 15 ml of water or phosphate buffer in a 15 cm porcelain mortar. The mortar was pre-chilled and kept in ice-water during the whole operation. The mixture was vigorously ground intermittently. During the intermission periods, the charge adhering to the wall and the stem of the pestle was scraped off with a polyethylene spatula. The mixture was very tacky, but became less so after the first five minutes. At the same time, a strong thiamine-like odour was detected. Upon completion of grinding, the mixture was extracted with 40 ml of water or buffer depending upon the purpose of the experiment, by mixing and standing for about 1 hour at 0–5° C. It was then centrifuged at 20,000 $\times$ *g* for 60 minutes. The residue was re-extracted with water or buffer, using up to 40 ml. The combined extracts were pooled. A considerable amount of liquid was still retained by the residue. Electron microscopic examination of the residue showed less than 20 % intact cells remaining. However, the protein found in the extract was slightly less than 50 % of the weight of the cells used.

The foregoing cell-free extract was able to effect the following transformations:

1. ethanol → acetaldehyde
2. glycerol → dihydroxyacetone (DHA)
3. DHA → unknown products

* A preliminary report was presented before the 19th International Congress of Physiology, Montreal, Can., September, 1953. Published with the approval of the Monographs Publications Committee, Oregon State College, Research paper No. 237, Department of Chemistry.

This work was supported by the Nutrition Foundation, Inc., the Rockefeller Foundation, and the Division of Research Grants and Fellow-ships, National Institutes of Health, U.S. Public Health Service.

References p. 116.

4. glucose → unknown products
5. pyruvate → acetaldehyde
6. acetaldehyde → acetate

These are described in the following sections.

Oxidation of ethanol; alcohol dehydrogenase

Resting cells of *A. suboxydans* oxidize ethanol to acetic acid[1]. This oxidation has now been duplicated in cell-free extracts, and the DPN-specific alcohol dehydrogenase of this organism has been purified to a high degree. Details of the purification, which were similar to those employed by RACKER[9] for yeast alcohol dehydrogenase, follow.

All steps were performed at 0–10° C, except those especially mentioned: 180 ml of cell-free extract made from 0.05 *M* phosphate buffer at pH 6.0 was adjusted to 8.5 with cold dilute NaOH. The mixture was heated to 50–53° C for about 15 minutes. A small amount of precipitate was centrifuged off at ca. 20,000 × *g*. One hundred and eighty-five ml of the supernatant were mixed with 92.5 ml of cold acetone in a dry-ice bath. The precipitate formed was centrifuged off at high speed and discarded. The supernatant, which was still very viscous, was slowly mixed with 150 ml of cold acetone at 0 to —5° C. A precipitate was formed, which was centrifuged and resuspended in about 40 ml of water. The suspension was dialyzed for 3 hours against distilled water with six transfers. The remaining precipitate was centrifuged and discarded. Thirty ml of the supernatant were mixed with 10.8 g $(NH_4)_2SO_4$.

The precipitate was centrifuged and resuspended in 10 ml water. The small amount of insoluble material was centrifuged off. To the clear supernatant, 2 g of $(NH_4)_2SO_4$ were added. The precipitate that formed was centrifuged at high speed and discarded. The supernatant was further mixed with 2.3 g of $(NH_4)_2SO_4$ over a period of about 4 hours, and left at 0° C overnight. The precipitate thus formed was not crystalline, but was completely soluble in 1 ml of water. The solution was precipitated by addition of 2 ml of saturated $(NH_4)_2SO_4$ solution. This precipitation was repeated once. Finally the precipitate, still amorphous, was dissolved in about 5 ml of water. The solution was dialyzed in a tilted bag attached to a slowly rotating stirring motor, set at an angle of about 20° from the vertical. The bath contained two liters of distilled water, which was transferred twice during the dialysis. The resulting solution was lyophilized. A white fluffy powder was obtained, which possessed 14,000 Racker units per mg, as listed in Table I.

TABLE I

Purification of alcohol dehydrogenase from *A. suboxydans*

	Protein content mg	*Total units*	*Specific Activity Units/mg*
Crude extract, 180 ml	3,200	1,440,000	450
After acetone precipitation, 40 ml	400	960,000	2,400
Final preparation	9.6	134,400	14,000

The system contained 0.01 *M* ethanol, $5 \cdot 10^{-5}$ *M* DPN, 0.01 *M* sodium pyrophosphate, and the proper amount of enzyme in 0.1 % serum albumin (added as a protective colloid). pH, 8.4. The other conditions and the definition of the unit of activity were the same as previously used[9].

The acetaldehyde formed from ethanol oxidation was characterized as the 2.4-dinitrophenylhydrazone derivative. An authentic sample melted at 164° (uncorrected) the oxidation product at 164°, and the mixed melting point was 163°.

The final preparation was devoid of dehydrogenase activity for lactic acid, glycerol, acetaldehyde, DHA, glucose, sorbose or sorbitol.

Non-utilization of acetate

Previous efforts to demonstrate metabolism of exogenous acetate by *A. suboxydans*

References p. 116.

have been unsuccessful[1], in that no evidence for citrate formation or sulfanilamide acetylation could be observed. Sulfanilamide acetylation was therefore sought *during* the oxidation of ethanol by resting cells in phosphate buffer, but this also failed even in the presence of added coenzyme A (CoA) (*cf.* Table II). In these experiments, acetylation occurred only when adenosine triphosphate (ATP), CoA and the pigeon liver acetylation enzyme[10] were present. Other experiments with cell-free extracts of *A. suboxydans* gave the same negative results. It is thus evident that no acetyl phosphate or "active acetate" (presumably acetyl CoA) is produced in the oxidation of ethanol Lack of formation of these compounds is probably the principal reason for the failure to acetylate sulfanilamide or form citrate. The independence of phosphate in ethanol oxidation in *A. suboxydans*[1] is in line with these observations, and serves to further distinguish this oxidation from that found in *Clostridium kluyveri* or *Escherichia coli*, where acetyl phosphate and/or acetyl CoA are regularly produced[11,12].

TABLE II

Acetylation experiments with resting *A. suboxydans* cells

Flask no.	Substances added						μg SAM acetylated
	Alcohol	ATP	Acetate	Liver enzyme	Co A	A. sub. Cells	
1	+	—	—	—	—	+	0
2	+	—	—	+	+	+	0
3	+	—	+	+	—	+	0
4	+	+	+	+	—	+	5
5	—	+	+	+	+	+	72
6	—	+	+	+	+	—	60
7	—	+	+	+	—	—	0
8	+	—	—	+	+	+	0
9	—	+	+	—	+	—	0
10	—	+	+	—	+	+	0

The amounts used were: alcohol, 50 μmols; ATP, 3 mg; acetate, 50 μmols; liver enzyme (cf. 10), 0.6 ml; CoA, about 3 units, together with 20 μmols of cysteine; resting cells of *A. suboxydans*, 10 mg dry weight. The following constituents were common to all flasks: potassium phosphate, pH 7.0, 240 μmols; sulfanilamide, 10 μmols; $MgCl_2$, 10 μmols; 0.2 ml 10% KOH in the center well; water to 3.0 ml. Time = 100 minutes. Temp. = 30° C. At the end of the experiment, 1 ml of 25% TCA was added.

Glycerol oxidation

Previous work with resting cells[1] has revealed that *A. suboxydans* can oxidize glycerol through DHA with a consumption of four atoms of oxygen per molecule. This has been increased to five atoms in the present work, where the oxidation is allowed to proceed for five or more hours. Endogenous oxidation during these periods was practically zero, although sudden increases were occasionally noted after ten hours.

Using 400 μmols of glycerol in 125 ml Warburg flasks, the media were examined for products of metabolism after several hours of oxidation by resting cells. A suitable deproteinizing agent (trichloroacetic acid or a $ZnSO_4$-NaOH mixture) was added, and the mixtures were centrifuged. The supernatants were retained for study. On the average, about 700 μmoles of CO_2 were produced, indicating that decomposition proceeded somewhat beyond a single scission into a C_1 and C_2 unit. About 60 μmols of volatile acid were formed, together with about 80 μmols of reducing substances (in terms of

References p. 116.

DHA). No evidence could be obtained for the presence of glycolic or glyoxylic acids[13] or oxalic acid either through colorimetric test of the supernatant[13] or by precipitation with Ca^{+2} and titration with $KMnO_4$. Diacetyl and acetylmethylcarbinol (acetoin; AMC) were also judged absent[14,15], but another carbonyl-type material was observed, as follows: The oxidation mixture after $ZnSO_4$-NaOH deproteinization was evaporated to dryness under reduced pressure at temperatures below 50°. The dry residue was extracted with alcohol. The extract was evaporated to dryness *in vacuo*, and the residue was redissolved in water and heated with dinitrophenylhydrazine reagent for 1 hour[14,15]. About 10 mg of a crude orange-red product was obtained from 400 μmols of glycerol. The compound was washed with dilute hydrochloric acid and water[15], then reprecipitated three times from pyridine and twice from methanol. The product discoloured slightly on heating but did not melt or decompose up to 320° C.

The boiling points of some logical intermediates of DHA dissimilation are given in Table III, together with some characteristics of the 2.4-dinitrophenylhydrazones of these compounds and the unknown product. Absorption spectra of these derivatives are evidently too similar to permit satisfactory characterization. From the melting points of the derivatives, diacetyl, acetoin, and methylglyoxal are evidently excluded from possible identity with the unknown, and glyoxal is excluded on the basis of its volatility. The new compound was negative to the creatine-sodium carbonate test for acetoin or diacetyl. In view of the nonvolatility of the unknown with steam, its structure is being sought further among more highly oxygenated C_2, C_3 and C_4 compounds. Other highly polar carbonyl compounds, such as hydroxypyruvic acid or glycerol-α and β-phosphates, were relatively unaffected by *A. suboxydans*.

TABLE III

PHYSICAL CONSTANTS OF SOME INTERMEDIATES IN CARBOHYDRATE METABOLISM AND AN UNKNOWN PRODUCED BY *A. suboxydans*

Compound	*Boiling point, °C*	*2.4-dinitrophenylhydrazones*	
		Melting point, °C	*Absorption peak, λ* mμ*
Diacetyl	88	315**, ***	540
Acetoin	148	315**	540
Methylglyoxal	72+	299–300 (21)	550
Glyoxal	50	328 (22)	
Unknown	non-vol.++	> 320	565

* In 0.3 % sodium ethoxide in ethanol, determined in Beckman spectrophotometer, model B; wavelength not calibrated.
** Bis-2,4-dinitrophenylhydrazone.
*** Prepared and recrystallized under the conditions described in text for the unknown derivative.
\+ Begins to boil at 72°, but polymerizes at room temperature[21].
++ Less volatile than water.

When glycerol and DHA were oxidized by cell-free extracts, oxygen consumption was slow and less extensive than with whole resting cells. In a five hour period, about 2 and 1 atom of oxygen per molecule of each substrate were consumed, respectively. Addition of the following substances, either alone or in combination, failed to improve the oxidation: protogen concentrates, DPN, TPN, flavin adenine dinucleotide, cytochrome C, ATP, adenylic acid, diphosphothiamine, yeast extract, liver extract, or a

References p. 116.

boiled extract of alumina-ground *A. suboxydans* cells. The lowered oxygen uptake in cell-free extracts may possibly be due to impairment of electron transfer systems. Attempts are being made to examine these systems further, using triphenyltetrazolium chloride, methylene blue and other redox dyes.

Oxidation of glucose and Krebs cycle intermediates

The oxidative behaviour of the cell-free extract toward glucose is shown in Fig. 1. The net oxygen consumption was approximately 2 atoms per molecule of glucose after four hours oxidation (compared to over 6 atoms with resting cells). Similarly, KATZNELSON AND TANENBAUM[16,17] found that only 3 atoms of oxygen were consumed per molecule of glucose in cell-free extracts of *Acetobacter melanogenum*, while more complete

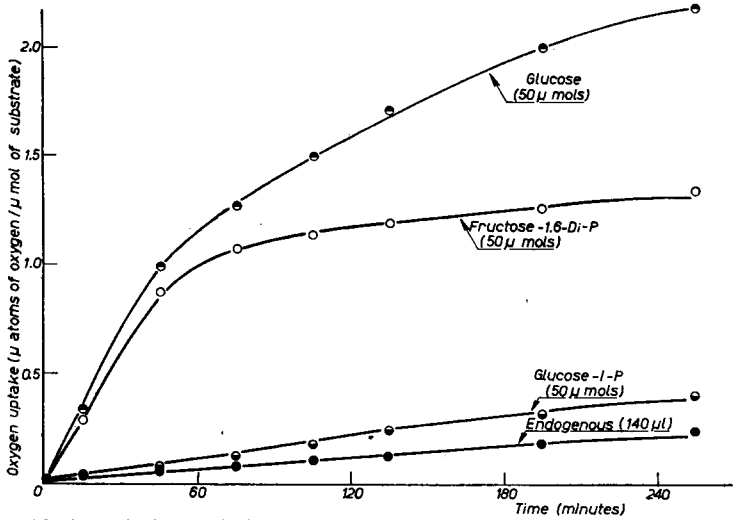

Fig. 1. The oxidation of glycolysis intermediates by cell-free extracts of *A. suboxydans*. System: 50 μmols substrate; 2.5 mg DPN; 1 mg cytochrome C; 15 μmols $MgCl_2$; 0.5 ml boiled extract of the supernatant of disintegrated cells (10 mg protein per ml), 1 mg ATP; 200 μmols of phosphate buffer and 15 mg (in terms of protein) of a cell-free extract; pH = 6.0; total volume = 2.8 ml; 0.2 ml of 10% KOH in the center well.

oxidation was found in intact cells. The slow oxidation of some glycolytic intermediates (*cf.* Fig. 1) both in intact cells and in cell-free extracts, may indicate the absence of glycolysis in the organism.

The intermediates of the tricarboxylic cycle were inert either toward resting cells or the soluble fraction of the disintegrated cells. The results are shown in Table IV. The oxidation was not improved by addition of the particulate fraction to the system. It is thus unlikely that the non-utilization of tricarboxylic cycle intermediates is due to permeability or to the retention of the enzymes in the insoluble fraction. The observed oxidation of oxalacetic acid was probably due to decarboxylation and subsequent oxidation of the pyruvate or acetaldehyde formed. It has been shown[18] that *A. suboxydans* contains an active pyruvic carboxylase and aldehyde dehydrogenase. The conversion of both of these substrates to acetate proceeds in the fashion depicted in Fig. 2. Also, oxalacetic acid was decarboxylated non-enzymically to an extent of about 2 μmols per 15 minutes from a similar system in the presence of a boiled extract of the disintegrated cells.

References p. 116.

TABLE IV

OXIDATION OF INTERMEDIATES OF TRICARBOXYLIC CYCLE BY A CELL-FREE EXTRACT OF *A. suboxydans*

Substrate	*Oxygen consumption (μ atoms)*
Fumaric acid	0.6
Succinic acid	0
Citric acid	14
Malic acid	0.3
Oxalacetic acid	17
α-Ketoglutaric acid	0.2
Acetic acid	0
Glycerol	51

The system contained 40 μmols of substrate; 1.7 mg TPN; 2.5 mg DPN; 1 mg cytochrome *c*; 15 μmols $MgCl_2$; 0.5 ml of boiled extract of the supernatant of the disintegrated cells; 120 μmols of phosphate buffer pH 6.0; and 15 mg (protein) of cell-free extract. Total volume = 2.8 ml; 0.2 ml of 10% KOH in the center well. Time = 270 minutes.

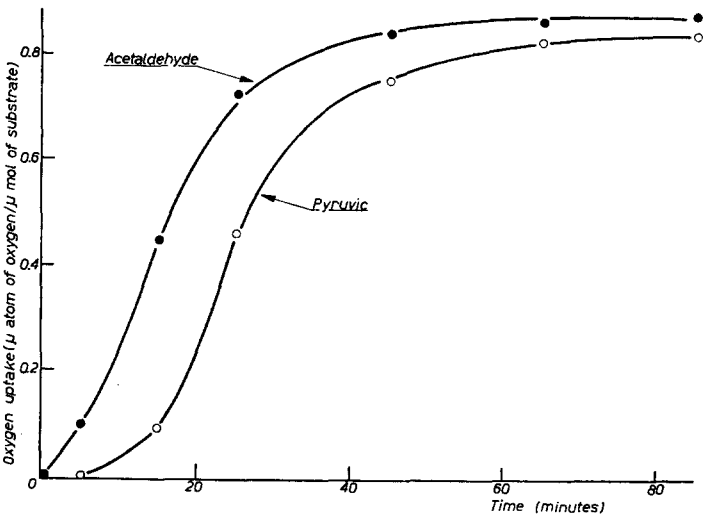

Fig. 2. Oxidation of pyruvic acid and acetaldehyde by cell-free extract of *A. suboxydans*. System: 60 μmols substrate; 20 μmols $MgCl_2$; 1 mg DPN; 0.5 mg co-carboxylase; 180 μmols phosphate buffer, pH 6.0, and 10 mg (protein) of cell-free extract; total volume = 2.8 ml; 0.2 ml of 10% KOH in the center well.

Non-enzymic combination of DPN and DHA

In the testing of alcohol dehydrogenase upon DHA, a non-enzymic reaction between DHA and DPN at high pH was observed. The results as shown in Fig. 3 indicated that the increase of optical density at 340 mμ was due to a non-enzymic reaction. The rate of the reaction was constant during at least the first two hours, using $5 \cdot 10^{-4}$ *M* DPN solution with an excess of DHA. The absorption spectrum of the resulting mixture was identical with that of the reduced DPN obtained from alcohol dehydrogenase and from hydrosulfite reduction. The aqueous solution of DHA in the absence of DPN was stable under similar conditions although concentrated DHA solution showed some absorption at shorter wavelengths. No reaction was observed when DPN was mixed with buffer in the absence of DHA.

References p. 116.

At pH values below 8.5, pyrophosphate buffer was superior to tris-(hydroxymethyl)-aminomethane and alanine buffers, whereas at high pH, no special effects were produced by the buffers. The reaction was pH sensitive, as shown in Fig. 4. However, whereas the equilibrium constant of the alcohol dehydrogenase-catalyzed reduction of DPN showed an expected 10-fold change with a shift of 1 pH unit, attempts to demonstrate a similar pH behaviour in this non-enzymic reaction were unsuccessful. It was thought that the reaction was a reduction[19], although the product failed to be oxidized by acetaldehyde in the presence of alcohol dehydrogenase. The latter fact had been considered

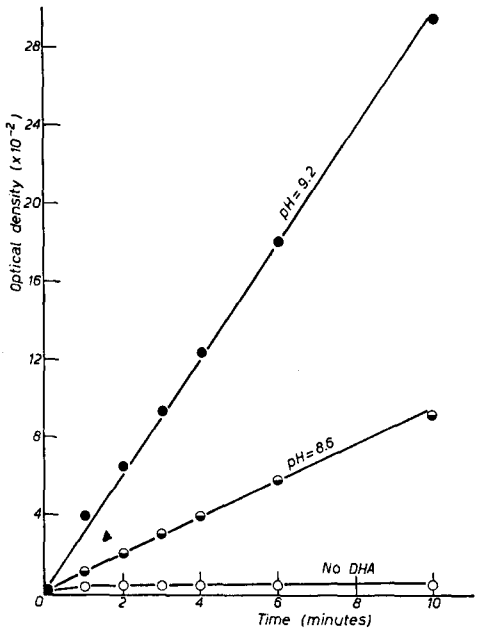

Fig. 3. Non-enzymic reaction of DPN with DHA, measured at $\lambda = 340$ mμ. System: 0.01 M pyrophosphate buffer and 0.05 % DPN, at indicated pH. DHA was added at zero time to 0.01 M concentration.

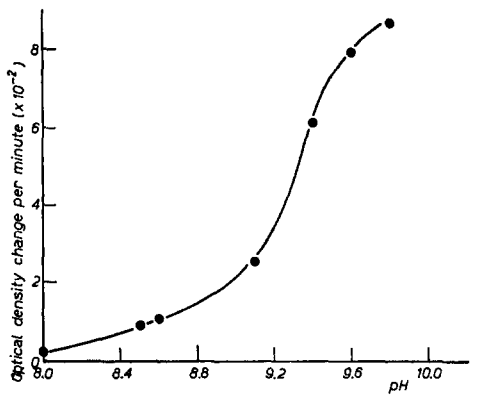

Fig. 4. The effect of pH on the rate of the non-enzymic reaction of DPN with DHA, measured at $\lambda = 340$ mμ. System: as in Fig. 3. The readings were the average values for the first ten minutes.

possibly due to an unfavourable pH value for the reverse reaction. More recently, BURTON AND KAPLAN[20] have also observed this reaction, and have found that it is not a reduction, but rather a formation of an addition complex between DPN and DHA. This reaction thus introduces a need for caution in interpreting spectrophotometric data obtained with systems containing pyridinoproteins and glycerol or DHA, particularly at high pH values.

ACKNOWLEDGEMENT

The technical assistance of GWENDOLYN CAHILL and MARION MACDONALD is gratefully acknowledged.

References p. 116.

SUMMARY

1. Cell-free extracts have been prepared from *A. suboxydans*, which are capable of catalyzing the following reactions:

ethanol → acetaldehyde
glycerol → dihydroxyacetone → a new carbonyl-type compound and other unknown products
glucose → unknown products
pyruvate → acetaldehyde
acetaldehyde → acetate

2. Ethanol dehydrogenase has been prepared in highly purified form (14,000 Racker units per mg). It is DPN-dependent.

3. The cell-free extracts (as well as intact resting cells) were unable to appreciably dissimilate acetate or intermediates of the tricarboxylic acid cycle. Glycolysis intermediates were also inert, and it is concluded that neither glycolysis nor the TCA cyclic oxidation pathway is operative to any significant degree in this organism.

4. A non-enzymic interaction between dihydroxyacetone and DPN is described. The product shows the same absorption behavior as reduced DPN, although it cannot be reoxidized by acetaldehyde.

RÉSUMÉ

1. Les auteurs ont préparé des extraits acellulaires de *A. suboxydans*, capables de catalyser les réactions suivantes:

ethanol → acétaldéhyde
glycérol → dihydroxyacétone → un nouveau corps carbonylé et d'autres produits inconnus
glucose → produits inconnus
pyruvate → acétaldéhyde
acétaldéhyde → acétate

2. L'éthanoldéshydrogénase a été obtenue sous une forme très purifiée (14,000 unités Racker par mg). Elle exige la présence de DPN.

3. Les extraits acellulaires (ainsi que les cellules intactes au repos) sont incapables d'utiliser d'une façon appréciable l'acétate ou les intermédiaires du cycle tricarboxylique. Les intermédiaires de la glycolyse sont également inertes et l'on peut en conclure que ni la glycolyse ni le cycle tricarboxylique n'interviennent d'une façon importante chez cet organisme.

4. Une interaction non enzymatique entre la dihydroxyacétone et le DPN est décrite. Le produit obtenu présente la même absorption que le DPN réduit, quoiqu'il ne puisse pas être réoxydé par l'acétaldéhyde.

ZUSAMMENFASSUNG

1. Es wurden zellfreie Extrakte von *A. suboxydans* hergestellt, die fähig sind folgende Reaktionen zu katalysieren:

Äthanol → Acetaldehyd
Glycerin → Dioxyaceton → eine neue Ketoverbindung and andere unbekannte Produkte
Glucose → unbekannte Produkte
Brenztraubensaures Salz → Acetaldehyd
Acetaldehyd → Acetat

2. Äthanoldehydrogenase wurde in höchst gereinigter Form dargestellt (14,000 Rackereinheiten/mg). Sie ist DPN-abhängig.

3. Die zellfreien Extrakte sowie die intakten verbleibenden Zellen waren unfähig Acetat oder Zwischenprodukte des Tricarbonsäurecyclus nennenswert zu dissimilieren. Glycolysezwischenprodukte reagierten ebenfalls nicht und es wird gefolgert, dass weder der Reaktionsverlauf der Glykolyse noch der TCS-Cyclusoxidation in diesem Organismus in bedeutenden Ausmass wirksam ist.

4. Eine nicht enzymatische Reaktion von Dioxyaceton and DPN wird beschrieben. Das Produkt zeigt das gleiche Absorptionsverhalten wie reduzierte DPN, obwohl est nicht wieder von Acetaldehyd oxydiert werden kann.

References p. 116.

REFERENCES

1 T. E. KING AND V. H. CHELDELIN, *J. Biol. Chem.*, 198 (1952) 127.
2 H. MICKLE, *J. Roy. Microscop. Soc.*, 68 (1948) 10.
3 W. W. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Manometric Methods*, Burgess, Minneapolis (1949).
4 E. R. STADTMAN AND H. A. BARKER, *J. Biol. Chem.*, 180 (1949) 1085.
5 W. P. WIGGERT, M. SILVERMAN, M. F. UTTER AND G. H. WERKMAN, *Iowa State Coll. J. Sci.*, 14 (1940) 179.
6 I. C. GUNSALUS AND W. W. UMBREIT, *J. Bact.*, 49 (1945) 347.
7 M. MCILWAIN, *J. Gen. Microbiol.*, 2 (1948) 288.
8 O. HAYAISHI AND R. Y. STANIER, *J. Bact.*, 62 (1951) 691.
9 E. RACKER, *J. Biol. Chem.*, 184 (1950) 313.
10 N. O. KAPLAN AND F. LIPMANN, *J. Biol. Chem.*, 174 (1948) 37.
11 E. R. STADTMAN AND H. A. BARKER, *J. Biol. Chem.*, 180 (1949) 1095.
12 S. OCHOA, J. R. STERN AND M. C. SCHNEIDER, *J. Biol. Chem.*, 193 (1951) 691.
13 V. P. CALKINS, *Ind. Eng. Chem., Anal. Ed.*, 15 (1943) 762.
14 C. NEUBERG AND E. STRAUSS, *Arch. Biochem.*, 7 (1945) 211.
15 F. C. HAPPOLD AND C. P. SPENCER, *Biochim. Biophys. Acta*, 8 (1952) 18.
16 H. KATZNELSON AND S. W. TANENBAUM, *Federation Proc.*, 12 (1953) 470.
17 H. KATZNELSON, S. W. TANENBAUM AND E. L. TATUM, *J. Biol. Chem.*, 204 (1953) 43.
18 T. E. KING AND V. H. CHELDELIN, Abstr. Comm., 19th International Physiological Congress, Montreal, Canada, 517 (1953).
19 T. E. KING AND V. H. CHELDELIN, Abstracts, 123rd Amer. Chem Soc. Meeting, Los Angeles (1953) 38 C.
20 R. M. BURTON AND N. O. KAPLAN, *J. Am. Chem. Soc.*, 75 (1953) 1005.
21 I. M. HEILBRON AND H. M. BUNBURY, Dictionary of Organic Compounds, 3, Oxford, New York (1938).
22 R. L. SHRINER AND R. C. FUSON, The Systematic Identification of Organic Compounds, New York (1948).

Received December 11th, 1953