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Carbohydrate Metabolism in *Spirochaeta recurrentis*

3. PROPERTIES OF ALDOLASE IN SPIROCHAETES*

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The enzyme aldolase, which catalyses the reversible splitting of fructose diphosphate, is of universal distribution amongst those living things which catabolize glucose by the Embden-Meyerhof route (Stumpf, 1954). In fact, since aldolase constitutes a 'key' enzyme in the reactions of anaerobic glycolysis its existence has been used as an indication of the presence of this pathway in various micro-organisms (Gunsalus, Horecker & Wood, 1955).

Recently, interest has been focused upon properties of the enzyme illustrated by the work of Rose & Rieder (1958) and the studies of Krishna Murti (1958), which have been concerned respectively with the mechanism of aldolase action and with its metal-ion activation in muscle, yeast and bacteria. Aldolase activity has been demonstrated in *Spirochaeta recurrentis* (Smith, 1960) and, as nothing is known about this enzyme in spirochaetes, a general study was made of its properties. On the results of work reported here, evidence is presented for the existence of a metallo-aldolase in this micro-organism.

EXPERIMENTAL

Methods

Isolation of spirochaetes from the blood of infected rats and preparation of cell-free extracts have been described by

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Smith (1960), as have the methods for determination of extinction, nitrogen and protein.

Abbreviations. The following abbreviations have been used in this paper: FDP, D-fructose 1:6-diphosphate; EDTA, ethylenediaminetetra-acetate (sodium salt); PCMB, p-chloromercuribenzoate.

Aldolase assay. The purified enzyme prepared from rabbit-skeletal muscle, which was used in FDP determinations, was assayed by the Warburg & Christian (1943) procedure as modified by Taylor (1955).

With crude extracts optical assay cannot be relied upon to provide accurate information, and therefore aldolase activity of spirochaetal extracts was measured by the Bard & Gunsalus (1950) modification of the Sibley & Lehninger (1949) technique as described by Smith (1960). The activity of the extracts was expressed in $\mu\text{g. of alkali-labile phosphorus produced/hr./mg. of protein by using the factor } 10.8$, after the manner of Bard & Gunsalus (1950) for an exactly similar assay system calibrated with purified FDP.

pH measurements. Determination of optimum pH of the spirochaete aldolase was carried out in veronal-acetate buffer (Michaelis, 1931), solutions being checked before use with a meter (Muirhead Type D-417, Muirhead and Co. Ltd., Beckenham, Kent, England) and glass electrode.

Inhibition and activation experiments. Tube contents were pre-incubated in the presence of various inhibitors, and for this purpose EDTA, 8-hydroxyquinoline, $\alpha\alpha'$ -dipyridyl and PCMB were added to the aldolase-assay tubes 10 min. before the substrate. Bard & Gunsalus (1950) recommended that tests with dipyridyl be carried out at pH 6.0 because of the greater activity of the reagent as a Fe^{2+} -ion binder at acid pH, but owing to the finding that a fairly sharp alkaline pH optimum existed for *S. recurrentis* aldolase this was not possible. When using cysteine, glutathione or metal ions, alone or in combination, precautions were taken to allow for the complex-forming tendencies which

metals exhibit with amino acids or peptides (Gilbert, Otey & Price, 1951; Rabin, 1955). In addition, tubes in which metal ions and cysteine were present were gassed with hydrogen, as used by Jagannathan, Singh & Damodaran (1956), to avoid the atmospheric oxidation of Fe^{3+} and Co^{3+} ions.

Dialysis. Dialysis of diluted cell-free extracts was carried out in cellophan tubing (Visking-Nojax, U.S.A.), previously cleaned by boiling with water and soaking according to the method described by Klotz & Hughes (1956). To ensure thorough mixing of the contents glass beads were enclosed in the dialysis sacs.

Materials

General reagents were of AnalaR grade wherever possible. FDP was prepared by yeast fermentation, after the method of Neuberg & Lustig (1942), and purified as the magnesium salt (Neuberg & Sabatay, 1925). This salt was converted into the sodium salt as previously described (Smith, 1960). FDP samples were assayed optically with rabbit-muscle aldolase and glyceraldehyde 3-phosphate dehydrogenase (Cori, Slein & Cori, 1948). Reduced glutathione, dipyridyl, 8-hydroxyquinoline and EDTA (disodium) were commercial preparations. Cysteine hydrochloride and PCMB were gifts.

Fig. 1. Effect of pH on aldolase activity. Tubes contained: 1.5 ml. of veronal-acetate buffer, 0.14M, of pH shown; 0.25 ml. of 0.56M-hydrazine sulphate; 0.20 ml. of cell-free extract, diluted with 0.9% NaCl to contain approx. 25 μg. of protein; 0.20 ml. of FDP, 15 μmoles; water to 2.5 ml. Temp. 37°. Substrate was added at zero time. Incubated for 15 min. Blanks had the same contents as listed above, but substrate was added after 2 ml. of 10% (w/v) trichloroacetic acid had been used to stop the reaction.

RESULTS

Previous experiments (Smith, 1960) have shown that extracts of *S. recurrentis* contain an active diphosphofructoaldolase, and that enzymic activity is located within the soluble portion of the organism. These findings were confirmed and experiments extended to examine the following properties.

Enzyme concentration and activity. The rate of the aldolase-catalysed splitting of FDP was found to be linear over the range 10–60 μg. of protein. For this reason tests were arranged to contain from 10 to 60 μg. of protein/2.5 ml. of reaction mixture.

pH optimum. Experiments over the range pH 7.5–9.5 show that the optimum for *S. recurrentis* aldolase lies at or about pH 8.75 (Fig. 1). The activity rises sharply before the optimum and falls off less rapidly thereafter.

Effect of substrate concentration. Examination of the curve showing reaction velocity–FDP concentration (Fig. 2), reveals that under these experimental conditions reaction velocity was maximal at a substrate concentration of about 3 mM. An approximate value for the Michaelis constant derived from the experimental curve (Michaelis & Menten, 1913) is 1.4 mM. It must be emphasized that this value is an approximation, owing to the

Fig. 2. Effect of substrate concentration on aldolase activity. Tube contents were as described in Fig. 1, except that FDP concentration varied from 2 to 14 μmoles per tube. Velocity of the reaction was measured over 20 min. at pH 8.5 and at 37°. Blanks contained corresponding amounts of FDP, added after the reaction had been stopped with trichloroacetic acid.

unpurified nature of the extracts used. For the same reason demonstration of activity of the spirochaetal enzyme towards other substrates such as fructose 1-phosphate, as has been shown for liver aldolase (Leuthardt & Wolf, 1954), was not attempted.

Effect of metal-binders and activators on aldolase. The requirement of *S. recurrentis* aldolase for metal ions and the presence of intact sulphydryl groups for full activity was investigated by means of metal-complexing agents, sulphydryl reagents and a sulphydryl-group inactivator. Experiments were set up in which the enzyme was exposed to EDTA, 8-hydroxyquinoline, dipyridyl, or to cysteine, glutathione or PCMB (Hellerman, Chinard & Deitz, 1943) as detailed below. The bivalent metal ions Fe^{2+} , Co^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} also were used.

(a) *Effect of metal-binders and sulphydryl reagents.* Table 1 shows the effect on aldolase activity of the metal-ion chelators mentioned above. Included in this table is the effect of pre-incubation of the cell-free extract in the presence of PCMB, and of equivalent amounts of cysteine and glutathione. It can readily be seen that, of the chelating agents, EDTA, an efficient bivalent-cation-binder, inhibited most effectively, whereas 8-hydroxyquinoline and dipyridyl did so to a less extent. PCMB completely inhibited enzyme activity at both the concentrations used, and cysteine and reduced glutathione enhanced the activity considerably; this has been shown to be characteristic of sulphydryl-group-requiring enzymes (Madsen & Cori, 1956). From these results it was concluded that *S. recurrentis* aldolase is probably a sulphydryl enzyme requiring metal ions for full activity.

(b) *Effect of bivalent-metal ions.* The ions Fe^{2+} , Co^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} were investigated. These metals were chosen because of the role they frequently play in biological systems (Williams, 1953).

Solutions of the hydrated sulphates of the metals were prepared to give final concentrations of 0.4 mM; earlier tests revealed that double or treble this concentration was too great for the enzyme dilution used and made comparison difficult. Pre-incubation of the enzyme with the metal was a prerequisite for full effect. Table 2 (a) shows the results of a typical series of experiments in which the use of metal ions alone demonstrated that Co^{2+} and, to a lesser extent, Zn^{2+} ions activated the enzyme, whereas Fe^{2+} and Mn^{2+} ions were inhibitory. Mg^{2+} ions appeared neither to activate nor to inhibit.

Next (Table 2 b) a small amount of EDTA was used to chelate extraneous ions first, then a slight excess of each metal ion was added and the cell-free extract incubated in its presence before the addition of FDP. There was a well-defined activation by Co^{2+} and Zn^{2+} ions, with cobalt again exerting the greater effect. All other metals inhibited in this experiment.

(c) *Effect of metal ions plus cysteine.* The combined effect of metal ions and cysteine upon the system was tried. The order in which cell-free extract, metal ion and cysteine were added, and the quantities, were found to be of the greatest importance in obtaining reproducible results. In the system finally adopted the metal ion was added immediately after the extract and about 10 min. at 37° was allowed for activation to take place, the assumption being that the metal combines first with the protein, as reported by Malmström (1954)

Table 1. *Effect of metal-complexing agents and sulphydryl reagents on Spirochaeta recurrentis aldolase*

Tubes contained: 1.5 ml. of veronal-acetate buffer (0.14M), pH 8.5; 0.25 ml. of 0.56M-hydrazine sulphate, pH 7.6; 0.20 ml. of cell-free extract, diluted to contain 25 μg . of protein; 0.15 ml. addition, 0.2 ml. of FDP, 15 μmoles ; water to 2.5 ml. Tubes were pre-incubated at 37° for 10 min. after making additions. Reaction was started by adding FDP and allowed to proceed for 15 min. Appropriate blanks contained additions but no FDP. Colour development was by the usual method.

Additions	Concn. (mM)	Activity (μg . of alkali- labile phosphorus/ hr./mg. of protein)	Percentage of original activity
None	—	605	100
EDTA	1.6	43	7
EDTA	0.8	46	8
8-Hydroxyquinoline	1.6	173	29
8-Hydroxyquinoline	0.8	432	71
$\alpha\alpha'$ -Dipyridyl	1.6	259	43
$\alpha\alpha'$ -Dipyridyl	0.8	562	93
PCMB	1.6	0	0
PCMB	0.8	0	0
Cysteine	1.6	1510	249
Cysteine	0.8	993	164
Glutathione	1.6	1340	222
Glutathione	0.8	820	135

Table 2. *Effect of metal ions on Spirochaeta recurrentis aldolase*

(a) Metal ions alone. Tube contents were as given in Table 1; additions contained $1 \mu\text{mole}$ of metal salt. Extract was pre-incubated at 37° with metal ion for 10 min. before addition of substrate. Reaction was allowed to proceed for 15 min. at pH 8.5. Control contained no added metal.

Metal	Activity (μg . of alkali-labile phosphorus/hr./mg. of protein)
Control	545
Fe ²⁺	272
Co ²⁺	1080
Mg ²⁺	539
Mn ²⁺	408
Zn ²⁺	618

(b) EDTA plus metal ions. 1 μ mole of EDTA was added to the tubes first, then cell-free extract and then metal ion (2 μ moles). Incubated at 37° for 110 min. Control contained no added metal or EDTA.

Metal	Activity (μg of alkali-labile phosphorus/hr./mg. of protein)
Control	543
Fe^{3+}	403
Co^{2+}	1350
Mg^{2+}	272
Mn^{2+}	260
Zn^{2+}	1075
No metal	270

(c) Metal ions plus cysteine. 2 μ moles of metal were added to the tubes first, followed by cell-free extract. Tubes were incubated for 10 min. at 37°. Hydrogen was bubbled through the reaction mixture before addition of cysteine (6 μ moles). Control contained no added metal or cysteine.

Metal	Activity ($\mu\text{g. of alkali-labile phosphorus/hr./mg. of protein}$)
Control	530
Fe^{2+}	750
Co^{2+}	1955
Mg^{2+}	1575
Mn^{2+}	1140
Zn^{2+}	1215
No metal	1760

Table 3. *Dialysis of cell-free extracts of Spirochaeta recurrentis in the presence and absence of Co²⁺ and Zn²⁺ ions*

Tube contents, in duplicate, consisted of 3 ml. of cell-free extract diluted five times with ice-cold 0.9% NaCl, pre-incubated for 10 min. at 37° in the presence or absence of metal ions (5 mM). Solutions were then placed in dialysis sacs each immersed in 50 ml. of 0.9% NaCl, and rocked mechanically for 48 hr. at 2°. The 0.9% NaCl was changed once during this time. Undialysed controls were pre-incubated for the same length of time and also kept at 2°. Activity assays were then carried out.

Additions before dialysis	Activity (μ g. of alkali-labile phosphorus/hr./mg. of protein)	
	Undialysed	Dialysed
None	392	473
Co ²⁺	203	338
Zn ²⁺	470	470

for crystalline enolase. Hydrogen gas was then bubbled through the reaction mixture and three times as much cysteine as metal was added to activate the sulphhydryl groups of the enzyme. It was assumed that in this manner there would be sufficient of the metal ion adjacent to the enzyme, and ample cysteine available to provide activity even after chelation had taken place.

The results (Table 2c) do in fact show that in the presence of cysteine Co^{2+} ions give maximal activity and, making allowance, for controls without added metal and with cysteine alone, that Fe^{2+} , Mn^{2+} and Mg^{2+} ions inhibit in that order. Anomalously, zinc appears to inhibit although, as shown earlier, it can apparently activate the enzyme.

(d) Effect of dialysis on aldolase activity. Owing to the loss of activity exhibited by spirochaetal extracts on storage in the cold, a property also of clostridial extracts noticed by Bard & Gunsalus (1950), no clear-cut conclusions on the role of metal ions could be drawn from the results of dialysis experiments. It was noticed, however, that dialysed extracts appeared to retain their aldolase activity longer than undialysed ones. Pre-incubation in the presence of Co^{2+} ions resulted in a more rapid loss of activity on storage or dialysis than did pretreatment with Zn^{2+} ions. Lastly, in some way zinc appeared to preserve the activity of the undialysed extract. These results are summarized in Table 3.

DISCUSSION

It should be emphasized that all the results reported here were obtained by use of the chemical triose-trapping method of aldolase assay, which is not prone to the interfering side reactions involved in the use of coupled optical systems with crude

Table 4. *Properties of some aldolases from different sources*

Source	Assay system*	pH optimum	$10^5 K_m$ (M)	—, Indicates that no data were given.	
				Activators	Inhibitors
<i>Clostridium perfringens</i>	T	7.5	1	Fe ²⁺ , Co ²⁺ cysteine	$\alpha\alpha'$ -Dipyridyl, o-phenanthroline, pyrophosphate
<i>Clostridium butyricum</i>	T	7.6	1	Fe ²⁺ , Co ²⁺ cysteine	Fe ²⁺ , Mn ²⁺ , Ni ²⁺ , Cu ²⁺ , Zn ²⁺ , $\alpha\alpha'$ -dipyridyl, o-phenanthroline, pyrophosphate
<i>Pasteurella pestis</i>	T	8.6	1.3†	Co ²⁺ cysteine	Fe ²⁺ , Zn ²⁺ , Mn ²⁺ , o-phenanthroline, EDTA
<i>Brucella suis</i> BCG	T	7.0-7.5	—	Fe ²⁺ cysteine	$\alpha\alpha'$ -Dipyridyl, o-phenanthroline
<i>Aspergillus niger</i>	O	7.5	1.5	Zn ²⁺	Co ²⁺ , Ca ²⁺ , Cd ²⁺ , Fe ²⁺ , Fe ³⁺ , cysteine, phosphate
Yeast	O	—	—	Zn ²⁺ , Fe ²⁺ , Co ²⁺	Cysteine, EDTA, pyrophosphate, o-phenanthroline, $\alpha\alpha'$ -dipyridyl
<i>Spirocheta recurrentis</i>	T	8.75	1.4	Co ²⁺ , Zn ²⁺ , cysteine	Cysteine, $\alpha\alpha'$ -dipyridyl, pyrophosphate
<i>Trypanosoma cruzi</i>	T	7.3	1.2	—	Fe ²⁺ , Mn ²⁺ , $\alpha\alpha'$ -dipyridyl, 8-hydroxyquinoline, EDTA
<i>Trichomonas vaginalis</i> Muscle (rabbit)	T	7	1	Fe ²⁺ , Co ²⁺	Cu ²⁺ , pyrophosphate, 8-hydroxyquinoline
	T	9	1	No metal activation	Cu ²⁺ , Zn ²⁺ , $\alpha\alpha'$ -dipyridyl, EDTA Cu ²⁺ , Ag ⁺ , I ₂

* T, trapping trioses; O, optical.

† Derived from published data.

Reference

Bard & Gunsalus (1950)

Gavard (1954)

Srikantan, Krishna Murti &
Shrivastava (1958)Gary, Kupferberg & Graf (1955)
Su & Ling (1956)Jagannathan, Singh & Damodaran
(1956)

Warburg & Christian (1942)

Present investigation

Baernstein & Rees (1952)

Baernstein (1954)

Herbert, Gordon, Subrahmanyam
& Green (1940)

extracts. With pure enzyme preparations the optical assay is the method of choice, and by its means Baranowski & Niederland (1949) found a pH optimum of 6.7–7.5 for crystalline rabbit-muscle aldolase. These authors supposed that trapping by chemical means becomes incomplete at pH values below 9, thus simulating a decrease in the rate of aldolase activity. This may be the reason for the high value of pH 9 quoted by Herbert, Gordon, Subrahmanyam & Green (1940) and by Peanaskey & Lardy (1958), who used cyanide to trap the trioses formed in the muscle- and liver-aldolase reaction. There may be reason to believe that this is the case with hydrazine also, for *S. recurrentis* aldolase activity falls off sharply on either side of pH 8.5–9.0. However, Bard & Gunsalus (1950), Baernstein & Rees (1952), Baernstein (1954) and Srikantan, Krishna Murti & Shrivastava (1958) all used hydrazine as a trapping agent and were able to show different pH-dependent behaviour for their enzyme preparations. The general shape of the curve for *S. recurrentis* aldolase most closely resembles that of Bard & Gunsalus for *Clostridium perfringens*. Moreover, the optimum reported in the previous paper for the diphosphopyridine nucleotide-reducing system with FDP as substrate and catalysed by spirochaetal extract also lay between pH 8 and 9.

There is a close correlation, too, between the K_m 1.4 mM for FDP in the aldolase-catalysed reaction and the optimum FDP concentration in the diphosphopyridine nucleotide-reducing system. It seems probable therefore that the rate-limiting reaction in the system is the aldolase-catalysed splitting of FDP. The K_m value obtained here is close to the value of 1.3 mM derived from the experimental data of Srikantan *et al.* (1958) for the partially purified aldolase of *Pasteurella pestis*, examined under similar test conditions. Values for purified and unpurified aldolase from different sources have been obtained by various workers and a list of some of these is included in Table 4. Most of the K_m figures quoted are about 1 mM, depending upon the conditions of assay. One of the difficulties in drawing comparisons of this kind lies in variation in experimental parameters affecting the Michaelis constant (Dixon & Webb, 1958), so that often there is no common ground on which to compare differences.

The most striking character affecting the activity of aldolase is perhaps its behaviour towards metal ions. Depending upon its source, the enzyme either does or does not require the participation of metal ions for maximal activity. Herbert *et al.* (1940), for instance, showed that traces of heavy metals inactivated the crystalline enzyme from rabbit muscle. By contrast, the enzyme from yeast first isolated by Warburg & Christian (1942) does not

show full activity unless previously incubated in the presence of either bivalent zinc, cobalt, iron or copper ions. In this respect the muscle enzyme appears to stand alone, because aldolases from *Clostridium perfringens* (Bard & Gunsalus, 1950), *C. butyricum* (Gavard, 1954), *Aspergillus niger* (Jagannathan *et al.* 1956) and *Pasteurella pestis* (Srikantan *et al.* 1958) also show bivalent-metal activation; the clostridial and *Pasteurella* aldolases are, in addition, cysteine-activated (see Table 4).

In the experiments described above both cobalt and zinc were found to exert an activating effect upon the spirochaetal enzyme. With cysteine, however, zinc appeared to be inhibitory. This behaviour might be explained if it is tentatively assumed that cobalt is the natural activator of *S. recurrentis* aldolase, and that zinc is able to replace it under certain conditions. Both zinc and cobalt are readily chelated by cysteine (Albert, 1952), the association constant for zinc being somewhat higher than that for the other ion. Consequently if zinc were not so firmly bound to the enzyme it might be removed more easily by the cysteine. In addition, experiments showed that zinc was able to relieve the inhibition produced by EDTA, but not to such an extent as cobalt. If the above assumption is correct therefore, it is probable, because of the close similarity of Co^{2+} - and Zn^{2+} -EDTA-complex association constants (Martell & Calvin, 1952) that EDTA also is able to remove some of the zinc from the protein. By reason of its close similarity to cobalt in ionic radius and electro-negativity (Wold & Ballou, 1957) it seems hardly surprising that zinc can substitute for cobalt in the activation of *S. recurrentis* aldolase. In fact the data given in Table 4 show that a proportion of aldolases are activated by these metals.

The significance of the finding that metal ions are themselves an indispensable feature of the activity of the spirochaete enzyme is emphasized by recent work on the classification of plant and animal aldolases on the basis of their inhibition or non-inhibition with EDTA (Bertrand & De Wolf, 1958). These authors' results show that greatest metal-ion dependence appears to exist among the aldolases of the lower forms of life of both plant and animal kingdoms. This being the case, it is felt that closer attention to purification of the aldolase of *S. recurrentis*, and the enzyme from other sources, may reveal the presence of a single-enzyme single-metal relationship similar to that existing amongst the zinc metallo-dehydrogenases.

SUMMARY

1. Some of the properties of a diphosphofructo-aldolase in cell-free extracts of *Spirochaeta recurrentis* have been studied.

2. By the hydrazine triose-trapping method, a pH optimum of 8.75 and an approximate K_m 1.3 mM have been demonstrated. These values are similar to those derived for the enzyme from other sources.

3. The enzyme is activated by Co^{2+} and Zn^{2+} ions, cysteine and reduced glutathione. Fe^{2+} , Mn^{2+} and Mg^{2+} ions either inhibit or have no effect. Ethylenediaminetetra-acetate, 8-hydroxyquinoline, $\alpha\alpha'$ -dipyridyl and *p*-chloromercuribenzoate inhibit aldolase activity. On the basis of these findings the spirochaetal enzyme is classed as a sulphhydryl-group-containing metalloaldolase.

4. The extent of metal-ion activation of different aldolases is briefly discussed.

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Carbohydrate Metabolism in *Spirochaeta recurrentis*

4. SOME PROPERTIES OF HEXOKINASE AND LACTIC DEHYDROGENASE IN SPIROCHAETES*

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The properties of enzymes occurring initially or terminally in a particular metabolic sequence often

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provide useful information about the rate-controlling processes which govern their activities in the intact organism (see, for example, Krebs, 1957; Krimsky, 1959; Holzer, 1959). Particular susceptibility to inhibition and hormonal influence is shown by hexokinase, the enzyme catalysing the