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

Table 1. *Effect of various additions on the hexokinase of Spirochaeta recurrentis under standard-assay conditions*

Reaction mixture contained: 0.1 ml. of KH_2PO_4 , 10 μmoles ; 0.1 ml. of EDTA, 8 μmoles ; 0.5 ml. of ATP, 20 μmoles ; 0.3 ml. of MgSO_4 , 45 μmoles ; 0.2 ml. of glucose, 160 μmoles ; 2.0 ml. of homogenate, approx. 10 mg./ml.; 0.1 ml. additions. Water to 4 ml. Reaction was started by addition of substrate. Temp. 35°. pH was maintained at 7.5 by addition of standard alkali (0.1 N-NaOH).

Additions	Final concn.	Activity (μmoles of glucose transformed/hr.)
None	—	30.6
Glucose 6-phosphate dehydrogenase + TPN ⁺	0.1 mg./4 ml. } 0.5 mg./4 ml. }	34.2
Fluoride	25 mM	0.4
Fluoride	12 mM	13.6
Fluoride	6 mM	35.5

primary phosphorylation of glucose by adenosine triphosphate (Lepage, 1950; Stadie, 1954). Similarly, in those organisms producing lactic acid from glucose, diphosphopyridine nucleotide-dependent lactic dehydrogenase may function as a metabolic regulator to an extent measurable by conventional methods of enzyme-kinetic analysis (Neilands, 1952; Nygaard, 1956). As both hexokinase and lactic dehydrogenase have been shown to be functional in *Spirochaeta recurrentis* (Smith, 1960), some of the properties of these two enzymes are reported, with special reference to their possible role in the carbohydrate metabolism of this parasitic spirochaete.

EXPERIMENTAL

Methods

Methods for the preparation of spirochaetal 'homogenates' (disintegrated cells) and cell-free extracts have been given in a preceding paper (Smith, 1960).

Abbreviations. DPN⁺, DPNH, oxidized and reduced forms of diphosphopyridine nucleotide; ADP, ATP, adenosine di- and tri-phosphate.

Hexokinase assay. After the photometric-indicator assays of Wajzer (1949) and of Crane & Sols (1955), and the methods of other authors (Kerly & Leaback, 1957; Slein, Cori & Cori, 1950), had been used initially without success, the assay finally adopted, because of its sensitivity and relative simplicity, was the micro-titrimetric constant-pH method (Schwartz & Meyers, 1958), which has already been described (Smith, 1960). Rate of formation of acid in micro-equivalents at neutral pH, after blank corrections, was taken to be equal to the rate of phosphorylation of glucose, which was expressed as μmoles of substrate consumed/hr./mg. dry wt. of homogenate.

Lactic-dehydrogenase assay. Routine assay of the pure crystalline commercial preparation, and of the activity of the enzyme in spirochaetal extracts, was performed in the manner described by Smith (1960).

The reaction in the reverse direction, with lactic acid as substrate, was carried out after the manner of Neilands (1955). In this, excess of lactic acid and of DPN⁺ were used in the presence of veronal-acetate buffer (0.14 M) (Michaelis, 1931) at pH 7.75. The glycine-NaOH buffer, pH 10,

recommended by Neilands, was not used for reasons given below.

Materials

General reagents were of AnalaR-reagent grade where possible and further purification was not attempted unless necessary. Pure crystalline sugars and D(+)-glucosamine hydrochloride were purchased from British Drug Houses Ltd. (Poole, Dorset). N-Acetyl-D-glucosamine was from L. Light and Co. Ltd. Sources of enzymes, coenzymes, sugar phosphates and sodium pyruvate have been given previously (Smith, 1960). Pure DL-lactic acid was prepared from commercial lactic acid (British Drug Houses Ltd.) via the zinc salt. Treatment of the zinc lactate with Zeo-Karb 225/H (The Permutit Co. Ltd., London) gave the free acid, from which sodium DL-lactate solution was obtained by neutralization with standard alkali. This is similar to the method used by Hakala, Glaid & Schwert (1956).

RESULTS

Hexokinase

Generally, activity was proportional to the quantity of spirochaetal homogenate added, and amounted to between 0.3 and 0.4 mg. of glucose consumed/hr./mg. dry wt. This is less than the glucose utilized by the whole spirochaetes (0.5–0.75 mg./hr./mg. dry wt.). Results varied from batch to batch, however, and, as activity fell rapidly after thawing, repeated experiments on the same homogenate were not always possible. Therefore experiments described here were of necessity limited by the labile nature of the homogenates themselves.

Effect of added zwischenferment and triphosphopyridine nucleotide. As inhibition by D-glucose 6-phosphate has been shown to occur with the hexokinase of rat-brain extracts by Weil-Malherbe & Bone (1951) and by Crane & Sols (1954), this product of the reaction was removed *in situ* by adding zwischenferment (glucose 6-phosphate dehydrogenase) and its coenzyme TPN⁺ to the reaction mixture. Table 1 shows that little increase in the activity was obtained, and the slowness of

the catalysis by spirochaetal hexokinase was unlikely therefore to be due to accumulation of glucose 6-phosphate. This experiment obviated also the chance that phosphofructokinase was contributing to acid production.

No glucose utilization occurred in the absence of ATP, but addition of larger amounts of ATP to the system did not increase the rate.

Effect of fluoride. Fluoride was added to the reaction mixture in some experiments to inhibit any adenosine triphosphatase present, and because Ochoa (1941) found difficulty in demonstrating the hexokinase of brain extracts in its absence. The precautions to be taken in using F^- ions in the hexokinase assay, however, where magnesium is required for optimum activity, have been emphasized by Melchior & Melchior (1956).

Addition of 100 μ moles of sodium fluoride to the reaction mixture before the start inhibited the activity completely. But, if addition was made after pre-incubation of the homogenate with ATP and Mg^{2+} ions, the inhibition was not so marked. However, in no instance did fluoride appear to enhance the activity of the spirochaetal enzyme (Table 1).

Substrate specificity. Table 2 shows the hexokinase activity of the homogenates in the presence

of various substrates. By far the greatest activity was shown with glucose, and then, in descending order, with mannose, sorbose, fructose and galactose. Even when allowance was made for its acid-binding power D(+)-glucosamine was not utilized at all, whereas it has been shown to serve as a substrate for the hexokinases from yeast (Brown, 1951; Grant & Long, 1952) and from brain (Sols & Crane, 1954). In fact, as shown below, this amino sugar apparently inhibited the utilization of glucose by spirochaetal homogenates.

Inhibition of hexokinase. Inhibition by glucosamine was found to be proportional to the amount added, as Table 3 shows. An extensive study of the inhibition of hexokinase by synthetic *N*-acyl derivatives of D-glucosamine (Maley & Lardy 1955) revealed that *N*-acetyl-D-glucosamine was highly specific, and inhibitory in a concentration as low as 1 μ M. The results obtained with this synthetic-substrate analogue showed that it, too, like the unsubstituted glucosamine, inhibited the hexokinase activity of *S. recurrentis* homogenates. To obtain maximal inhibition, however, it was necessary to add each inhibitor before the glucose, which suggests that, as with brain hexokinase (Sols & Crane, 1954), they compete with glucose for the enzyme surface.

Table 2. *Substrate specificity of Spirochaeta recurrentis hexokinase*

Reaction mixtures contained the same components as those described in Table 1, without extra additions. Equimolar amounts (160 μ moles/4 ml. of reaction mixture) of the substrate indicated were added, in 0.2 ml., to start the reaction. Temp. 35°. pH was maintained at 7.5.

Substrate	Activity	
	(μ moles/hr./mg. dry wt.)	(as percentage of glucose activity)
D(+)-Glucose	1.32	100
D(-)-Fructose	0.38	29
D(+)-Galactose	0.03	2
D(+)-Mannose	1.25	95
L(-)-Sorbose	0.61	46
D(+)-Glucosamine	0.00	0

Lactic dehydrogenase

Effect of extract concentration. Comparatively small quantities of cell-free extract brought about rapid oxidation of DPNH when pyruvate was used as substrate. The increase in velocity followed linearly the increase in the amount of extract added to the cuvette (Fig. 1).

Effect of pH. A comparison was made of the pH optima of purified muscle lactic dehydrogenase and that contained in *S. recurrentis* extracts. The activities of both enzymes were adjusted by dilution until nearly equal. When tests were conducted in veronal-acetate buffer at 20° the optimum for both muscle and spirochaetal enzymes was at pH 7.0, and the curves were closely similar in shape (Fig. 2).

Table 3. *Inhibition of glucose utilization in the Spirochaeta recurrentis hexokinase reaction*

Reaction mixtures contained the same components as those described in Table 1, except that additions were made before starting the reaction with glucose. Temp. 35°. pH was maintained at 7.5.

Inhibitor	Concn. (mm)	Activity	
		(μ moles/hr./mg. dry wt.)	Inhibition (%)
None	—	2.41	0
D(+)-Glucosamine	10	1.25	48
D(+)-Glucosamine	40	0.00	100
<i>N</i> -Acetyl-D-glucosamine	0.01	1.75	27
<i>N</i> -Acetyl-D-glucosamine	1.0	0.68	72
<i>N</i> -Acetyl-D-glucosamine	10	0.34	86

Fig. 1. Effect of extract concentration on lactic-dehydrogenase activity. Cuvettes contained: 2.65 ml. of triethanolamine buffer (0.05M), pH 7.5; 0.05 ml. of DPNH, 10 mg./ml.; 0.2 ml. of sodium pyruvate, 7 μ moles; cell-free extract containing, A, 220 μ g., B, 110 μ g., C, 70 μ g. of protein. Control, without substrate, tested for absence of DPNH-oxidase activity. Blank contained water. Reaction was started by addition of extract. Temp. 20°.

Fig. 2. Effect of pH on lactic-dehydrogenase activity. Cuvette contents: 2.7 ml. of veronal-acetate buffer (0.14M), of pH shown above; 0.05 ml. of DPNH, 10 mg./ml.; 0.2 ml. of sodium pyruvate, 7 μ moles; 0.05 ml. of extract, or pure lactic dehydrogenase suitably diluted, was added last to start the reaction. The blank contained water. Temp. 20°. O, Spirochaetal cell-free extract, 70 μ g. of protein; Δ , crystalline muscle lactic dehydrogenase, 0.5 μ g. of protein.

Effect of pyruvate concentration. Fig. 3 illustrates how the enzymic activity of the extracts responded to an increase in the substrate concentration. Activity reached a maximum at about 2.5 mM-pyruvate and then appeared to fall off slightly at higher concentration. The concentration of pyruvate corresponding to half-maximal velocity, the K_m of Michaelis-Menten kinetics (Michaelis & Menten, 1913), is approx. 0.3 mM. Owing to the unpurified nature of the enzyme being studied it was not considered justifiable to apply the more accurate Lineweaver & Burk (1934) treatment.

Reverse reaction, lactate oxidation. The glycine-NaOH buffer system used by Neilands (1955) at pH 10 gave a very low level of activity with spirochaete extract. After several buffers at different pH values had been tried, veronal-acetate buffer, pH 7.75, was used successfully. The curve shown in Fig. 4 is typical of the results obtained, with DL-lactate as substrate. The amount of DPN⁺ reduced during the oxidation of the lactate is comparatively small because the reaction rapidly reaches equilibrium.

Effect of lactate concentration. Kubowitz & Ott (1943) found that D(-)-lactate would not act as a substrate for muscle or tumour lactic dehydrogenase; nor will it interfere with the oxidation of the L(+)-isomer (Neilands, 1952). The majority of the lactic dehydrogenases from animal sources are stereospecific for L-lactic acid (Schlenk, 1951), whereas those of micro-organisms produce D-, L-

Fig. 3. Effect of pyruvate concentration on lactic-dehydrogenase activity. Cuvette contents were as described for Fig. 1, except that increasing concentrations of pyruvate were used, as indicated. Extract contained 35 μ g. of protein in 0.05 ml. used in tests. The blank contained water. Temp. 20°.

or DL-forms (Katagiri & Kitahara, 1937). It seemed highly probable that the spirochaetal enzyme also exhibits L-lactate specificity, because of the possibility that disposal of this metabolic end product of the parasite's metabolism takes place via the respiratory pathways of the host. Therefore it was tentatively assumed that the D(-)-lactate present in the substrate solution as prepared from DL-lactic acid would not interfere in the reaction catalysed by the enzyme from *S. recurrentis*, and concentration of substrate was calculated on the basis of the L(+)-lactate it contained, that is, half the amount of DL-lactate.

Examination of Fig. 5 reveals that maximal velocity is attained when the concentration of L(+)-lactate is 42 mM, which gives K_m about 10 mM with respect to L(+)-lactate. By comparing

Fig. 4. Lactate oxidation catalysed by *S. recurrentis* extract. Cuvette contained: 2.75 ml. of veronal-acetate buffer (0.14M), pH 7.75; 0.1 ml. of DPN, 20 mg./ml.; 0.1 ml. of sodium DL-lactate, 9 mg.; 0.05 ml. of cell-free extract, 100 μg. of protein. The blank contained water in place of substrate. Temp. 20°.

the K_m values for both forward and backward reactions it is apparent that the affinity of the enzyme for lactate is about one-thirtieth of that for pyruvate.

Equilibrium constant. Results obtained from equilibrium assays of the spirochaetal lactic dehydrogenase with lactate as substrate were used to calculate two approximate constants for the reaction equilibrium. No account was taken of the pyruvate kinase-catalysed phosphorylation of pyruvate to phosphoenolpyruvate, since the kinase was shown earlier to be relatively inactive in the absence of added ATP.

The concentration of DPNH was calculated from the extinction constants given by Horecker & Kornberg (1948), and the hydrogen term was omitted and affinity constants were assumed to be unity. The 'apparent' equilibrium constant ($K_{app.}$), derived from the figures quoted in Table 4, is 6.1×10^{-5} .

Fig. 5. Effect of L(+)-lactate concentration on lactic dehydrogenase activity. Cuvette contents were as described in Fig. 4, except that the concentration of lactate shown above, calculated on the basis of L(+)-lactate content, was used. Temp. 20°.

Table 4. *Equilibrium constants of the lactic-dehydrogenase reaction catalysed by Spirochaeta recurrentis extracts*

Measurement was made in veronal-acetate buffer (0.14M), pH 7.75, in a 1 cm. cell; 3 ml. total volume at 20°. I and II refer to two experiments with different concentrations of lactic acid.

Component	Initial concn. (M)		Final concn. (M)	
	I	II	I	II
L(+)-Lactic acid	13.5×10^{-3}	6.75×10^{-3}	13.4×10^{-3}	6.65×10^{-3}
DPN ⁺	9.5×10^{-4}	9.5×10^{-4}	9.2×10^{-4}	9.3×10^{-4}
Pyruvic acid	0	0	29.3×10^{-6}	17.9×10^{-6}
DPNH	0	0	29.3×10^{-6}	17.9×10^{-6}
		$K_{app.}$	7.0×10^{-5}	5.2×10^{-5}

K average = 6.1×10^{-5} .

Owing to the difficulty in obtaining sufficient experimental material no attempt was made to purify any of the enzymes of *S. recurrentis*, so that these values are approximate. Because of its relative stability on storage in the freeze-dried organisms, however, it is felt that purification of spirochaetal lactic dehydrogenase is a distinct possibility.

DISCUSSION

Attention has been drawn previously (Smith, 1960) to the finding that the hexokinase of *S. recurrentis* is associated with the particulate fraction of the organism. This, together with the unsuitability of hexose phosphates as substrates for the intact spirochaetes, points to the possibility that this hexokinase may be an ectoenzyme found on the cell surface, as is supposed to be the case with mouse ascitic-tumour cells (Kazakova, 1958). Soluble and particulate hexokinases have been known for some time (Slein *et al.* 1950; Crane & Sols, 1953), but they are not usually as difficult to demonstrate as was the spirochaetal enzyme. In fact, this difficulty, by comparison with the ease with which aldolase and lactic dehydrogenase were demonstrated, may be a manifestation of an inhibition significant in the control of *S. recurrentis* glycolysis.

The hexokinase reaction is known to be very sensitive to quite low concentrations of ADP (Lepage, 1950), the process of inhibition constituting a 'pacemaker' system, according to Krebs (1957). Also, kinetic studies by Beck (1955) on the rates of anaerobic glycolysis in human leucocytes have shown that although the functional capacity, V_{max} , of an individual enzyme may be demonstrably high, its actual performance in the metabolic chain may be low or limiting. These facts suggest therefore that in *S. recurrentis* the apparent slowness of the hexokinase reaction may reflect a mechanism whereby the speed of passage of the phosphorylated glycolytic intermediates through the Embden-Meyerhof pathway is regulated to make most economical use of the available substrate. Some measure of support is provided for this view by recent work of Randle & Smith (1958), showing that entry of glucose into muscle cells is normally the rate-limiting step in its metabolism.

Whether fructose is genuinely unsuitable as a substrate, or whether its measurably slower rate of phosphorylation by both whole and disintegrated spirochaetes is due to its enzymically inactive pyranose form in solution (Gottschalk, 1947), must await results of further inquiry.

From the limited investigation made of its properties, the lactic dehydrogenase of *S. recurrentis* must resemble its mammalian counterpart. The inhibitory effect of pyruvate at high concentra-

tions was first noticed by Kubowitz & Ott (1943) for their enzyme from rat muscle. But the spirochaetal enzyme K_m of 0.3 mM with pyruvate does not resemble the value for the muscle enzyme, which is variously quoted at 90 μ M (Kubowitz & Ott, 1943), 52 μ M (Meister, 1950), of the order of 10 μ M (Neilands, 1952) and 54 μ M (Kun, 1957). Constants for pyruvate six to twelve times as high as those for the mammalian-muscle enzyme have been found, however, for lactic dehydrogenase of *Schistosoma mansoni* by Mansour & Bueding (1954). Though no justification for far-reaching conclusions, these quantitative data on the *S. recurrentis* lactic dehydrogenase were thought to be of some value in assessing the relative importance of the terminal step in glucose catabolism by the spirochaete. For similar reasons, and to determine the direction favoured, investigation of the reverse reaction from lactate to pyruvate was carried out.

Comparison of the K_m for both forward and backward spirochaete lactic dehydrogenase-catalysed reactions showed that affinity for lactate was one-thirtieth of that for pyruvate. Neilands (1952) has shown that the K_m for heart-muscle lactic dehydrogenase at pH 7, 8, 9 and 10 was about 10 μ M for DPN⁺ and pyruvate. However, over the same pH range the K_m for L(+)-lactate was greater than 1 mM, that is, an affinity of about one-hundredth. The mean value for the equilibrium constant of the *S. recurrentis* enzyme is almost identical with the figure of 6.0×10^{-5} obtained for the rat and tumour enzyme by Kubowitz & Ott (1943). Both values are small, which means that the standard free-energy change (ΔF) has a large positive value and the reaction will not proceed far in the reverse direction spontaneously.

A possibly more important controlling influence in the lactic-dehydrogenase reaction can be presumed from its requirement for DPNH as a coenzyme. Any process tending to deplete sources of the pyridine nucleotide would result in an inhibition of the lactic-dehydrogenase-catalysed reaction. The gradual accumulation of pyruvic acid during the course of spirochaetal fermentation, far from being indicative of a steady-state equilibrium, could, indeed, reflect a metabolic inadequacy in the form of insufficient reduced coenzyme. Glock & McLean (1955) found that in all the animal tissues investigated the DPN⁺ was present mainly in the oxidized form, whereas Holzer, Schultz & Lynen (1956) found this to be the case in living yeast cells. Under certain conditions therefore terminal conversion of pyruvate into lactate in *S. recurrentis* might be governed by the availability of DPNH.

SUMMARY

1. The particulate hexokinase of *Spirochaeta recurrentis* shows maximum specificity towards

D-glucose and D-mannose. L-Sorbose and D-fructose may substitute for them to a less-marked extent. The enzyme is inactive with D-galactose and inhibited, it is assumed competitively, by D-glucosamine and N-acetyl-D-glucosamine. Fluoride does not enhance its activity.

2. Some of the properties also of lactic dehydrogenase have been studied in cell-free extracts of the organism. Under similar spectrophotometric-assay conditions both mammalian-muscle and *S. recurrentis* lactic dehydrogenases were optimally active at pH 7.0.

3. The conversion of pyruvate into lactate was investigated in the forward and backward direction. K_m values of 0.3 mM and 10 mM were obtained for pyruvate and L-lactate respectively. The approximate equilibrium constant for the reaction was 6×10^{-5} .

4. The significance of these findings and possible functions of the two enzymes as regulators of the carbohydrate metabolism of *S. recurrentis* are discussed.

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The Biosynthesis of Chondroitin Sulphate

INCORPORATION OF SULPHATE INTO CHONDROITIN SULPHATE IN EMBRYONIC CHICK CARTILAGE

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D'Abramo & Lipmann (1957) have demonstrated that active sulphate, adenosine 3'-phosphate 5'-sulphatophosphate is formed by an extract of embryonic-chick cartilage. It was further shown that adenosine 3'-phosphate 5'-sul-

phatophosphate was responsible for sulphation of chondroitin sulphate synthesized *in vitro* when the extract was incubated with adenosine triphosphate and magnesium chloride. After the isolation of the unsulphated mucopolysaccharide chondroitin