Little can be said about amino acid pattern other than that the proline-serine-threonine content is high, as in blood-group substances and cervical mucoids (see Table 2); but amino acids characteristically absent in these are present, with tyrosine in very large amounts.

SUMMARY

1. The nature of the mucoid comprising the substance of the lamellibranch crystalline style has been investigated in *Pinna nobilis* L.

2. It consists of approximately 30% of carbohydrate combined with 70% of protein. The carbohydrate portion is composed of *N*-acetylated glucosamine and galactosamine, galactose and fucose, which have been estimated. Although it resembles the blood-group substances in make-up, tests for A, B, H and Le^a activity were negative.

3. All the usual amino acids are present, with threonine-serine, proline and tyrosine in relatively large amounts.

4. By weight, the amount of enzyme protein (amylase, lipase, cellulase) is negligible, but the style mucoid itself, both electrophoretically and in the ultracentrifuge, behaves as a paucidisperse system.

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Carbohydrate Metabolism in *Spirochaeta recurrentis*

1. THE METABOLISM OF SPIROCHAETES IN VIVO AND IN VITRO*

BY J. D. FULTON AND P. J. C. SMITH†

National Institute for Medical Research, Mill Hill, London, N.W. 7

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In contrast with the intensive studies on bacteria, very few investigations have been carried out on the biochemical activities of the order Spirochaetales, whose three main divisions comprise *Spirochaeta* (*Borrelia*), *Leptospira* and *Treponema*. The review

* Part of a Ph.D. thesis submitted to the University of London.

† Present address: Medical Research Council, Rheumatism Research Unit, Manor Hospital, Bath, Somerset.

by Geiman (1952) on spirochaete metabolism emphasizes how little is known of this interesting group of micro-organisms. The intermediate taxonomic position of spirochaetes between bacteria and Protozoa, and the difficulty in cultivating pathogenic types *in vitro*, may account for this neglect.

Earlier workers (Fenyvessy & Scheff, 1930; Scheff, 1935) reported that *Spirochaeta recurrentis*

consumed glucose and produced lactic acid but did not use oxygen. McKee & Geiman (1950), using *Borrelia novyi* (another strain of relapsing-fever spirochaete), obtained similar results but indicated that there was a low consumption of oxygen. Comparable studies by Fulton & Spooner (1956) on *Leptospira icterohaemorrhagiae* showed the absence of a fermentative pattern of carbohydrate metabolism.

It was primarily because of the lack of any definite data upon the course of carbohydrate metabolism in spirochaetes that work on the biochemistry of *Spirochaeta recurrentis* was started. This paper serves as an introduction to further study.

METHODS AND MATERIALS

Parasite. The organism used for experiments both *in vivo* and *in vitro* was the tick-borne strain of *Spirochaeta recurrentis*, referred to in the literature by some authors either as *S. duttoni* or *Borrelia duttonii* (Breed, Murray & Smith, 1957), and was obtained from The Wellcome Laboratories.

Host. The strain was maintained throughout in albino rats weighing 70–80 g. Infected blood was obtained from two or three heavily infected donor rats by cardiac puncture under chloroform anaesthesia and mixed with 0.9% NaCl–2% (w/v) sodium citrate in the proportion of 1:1. A volume of 0.5–1 ml. of this mixture was inoculated intraperitoneally into other rats. The state of infection was judged daily by microscopical examination of tail blood. Peak infections of blood usually occurred 2 days after inoculation.

Experiments in vivo

Respiratory CO_2 in the rat host was measured in a metabolism cage based on that of McClintock & Lifson (1957), with modifications, so that samples of respiratory air could be taken at regular intervals and analysed for content of CO_2 by a simplified Haldane apparatus attached to the vessel in which the animal was housed. The vessel consisted of a desiccator, capacity 2 l., containing food bin, inlet and outlet for gases and one for drinking water. This arrangement avoided the tedious collection of large quantities of total CO_2 by the usual barium carbonate technique (Skipper, Bryan, White & Hutchinson, 1948).

Variations in the daily output of CO_2 by the rats under investigation were reduced to a minimum by taking samples between 10 a.m. and 4 p.m. each day, as recommended by Horst, Mendel & Benedict (1934). At the end of the first day the rat was removed from the apparatus, weighed and inoculated intraperitoneally with blood from another rat heavily infected with *S. recurrentis*. Analyses were then continued hourly throughout each day without removing the animal from the cage until the experiment was over. The rat was then taken out and weighed again.

Blood and liver samples from batches of normal and infected rats, for estimations of blood glucose, α -keto acids, lactic acid and liver glycogen, were always taken by cardiac puncture or excision of tissue immediately after killing by neck dislocation. Blood was run directly from the pipette into the protein precipitant to avoid variations caused by changes in the blood (Long, 1944), and liver samples were quickly weighed and plunged into the KOH solution used in the glycogen determination.

Experiments in vitro

Isolation of spirochaetes from blood. At the height of infection, when the blood was swarming with spirochaetes, the rats were anaesthetized by chloroform and bled by cardiac puncture into 0.9% NaCl–sodium citrate containing 0.2% of glucose. To this mixture was added 0.5 vol. of Krebs buffered medium (Krebs & Eggleston, 1940) at 37° containing 0.2% of glucose. After centrifuging at 250 g for 12 min. the cloudy supernatant was drawn off from the red blood cell residue and placed in 50 ml. plastic centrifuge tubes. The red blood cell layer was then washed twice more with the warm medium, centrifuged at 200 g for 10 min. and the supernatant also placed in plastic centrifuge tubes. The tubes were then centrifuged at 1500 g on an angle-head centrifuge for 4 min. exactly; the supernatant was discarded and a small amount of warm Krebs medium with glucose was added. On setting aside at a temperature of 30–35° the residue of motile spirochaetes begins to slide off the relatively tightly packed red and white blood cell layer and may be removed with a Pasteur pipette. This suspension was diluted further with warm 0.9% NaCl only and again centrifuged at 2000 g for 5 min. The thick layer of organisms was gently removed by agitating the 0.9% NaCl and the resultant suspension, which was essentially free of red and white blood cells, was used as such or diluted further with 5% (v/v) of rat serum in 0.9% NaCl containing 0.2% of glucose.

A curve correlating extinction, total nitrogen content (Kjeldahl) and dry weight (110°) was constructed. The density measurements were made on a Hilger Biochem Absorptionmeter with a neutral-grey filter (Ilford H 503).

Manometric measurements. For measurement of either O_2 or CO_2 gas exchange use was made of the conventional Warburg constant-volume manometric apparatus (Dixon, 1943; Umbreit, Burris & Stauffer, 1949) and also, to a limited extent, the modified Scholander-type differential constant-pressure respirometer (Smith, 1958). In some experiments acid production was measured in the presence of bicarbonate buffer and $\text{N}_2\text{--CO}_2$ (95:5) atmosphere (Umbreit *et al.* 1949).

Chromatography. Paper partition chromatography was utilized for identification of metabolic end products produced *in vivo* and *in vitro*, the various modifications of the original method of Consden, Gordon & Martin (1944) being detailed below.

Identification of organic acids. Before chromatography, the suspension of organisms, previously incubated with glucose, was deproteinized with sodium tungstate (5%, w/v) and sulphuric acid (0.6 N) and centrifuged. About 200 ml. of the deproteinized samples were neutralized with NaOH and concentrated *in vacuo* in a rotary evaporator. The solution was then acidified and distilled into dilute NaOH under slightly reduced pressure. The pH was adjusted to neutrality and the solution evaporated to near dryness. The residue of non-volatile material was also neutralized and evaporated to small volume.

The solution of salts of the volatile organic acids was acidified with an equal volume of orthophosphoric acid (60%, w/v) and the acids were obtained free from water by the method of James & Martin (1952a). The non-volatile acids were treated similarly. Solutions of the free acids were applied to paper and chromatographed with markers according to the procedure of Duncan & Porteous (1953) for volatile fatty acids, and that of Long, Quayle &

Stedman (1951) for the non-volatile acids. The zinc oxinate reagent of Pesze & Ferrero (1957) was also found useful in rendering the acids visible. The gas-liquid chromatography method of James & Martin (1952*a, b*) was also used in detecting volatile acids.

Identification of α -keto acids. Rats at the peak of infection were bled by cardiac puncture, heparin being used. After centrifuging the plasma was deproteinized and subjected to the Cavallini & Frontali (1954) procedure for the extraction of keto acids as 2:4-dinitrophenylhydrazones. These derivatives of pyruvic acid and α -oxoglutaric acid were used as markers and chromatographed by the technique of Altmann, Crook & Datta (1951).

Glycerol. For identification of glycerol, isolated by extraction of freeze-dried products by ethanol, chromatography was used as described by Grant & Fulton (1957).

Chemical methods

For reducing sugars the method of Nelson (1944) was used; for lactic acid that of Barker & Summerson (1941) as modified by Hullin & Noble (1953) was used, with lithium lactate as standard (Barker, 1957). Blood α -keto acid levels were measured by the method of Goodwin & Williams (1952), which was also used for estimating the production of pyruvic acid *in vitro* by the organisms.

^{14}C -Tracer studies. [Uniformly labelled (U)- ^{14}C] and [$1\text{-}^{14}\text{C}$]-glucose were used with suspensions of the live organisms and the distribution of radioactivity in the major products of metabolism *in vitro* was traced.

The final preparation of parasites from 25 rats was suspended in 15 ml. of Krebs buffered medium at 37°. Equal volumes (7 ml.) of the spirochaete suspension containing approx. 1.5 mg. dry wt./ml. were added to two volumetric flasks (50 ml.) each containing buffered medium + 0.3 % of glucose and about 0.75 μC of [U]- ^{14}C] or [$1\text{-}^{14}\text{C}$]-glucose. In a third flask, which served as a control, an equivalent quantity of serum from which spirochaetes had been removed by centrifuging replaced the spirochaete suspension and [U]- ^{14}C] glucose was present in the flask. After making up to 50 ml. with buffered medium the entire contents of each flask were tipped into three $\times 250$ ml. wide-mouthed conical flasks equipped with rubber-capped inlet and outlet tubes and a CO_2 -absorption well containing pleated filter paper (Whatman no. 40). The flasks were kept at 35° and gas, either $\text{N}_2 + \text{CO}_2$ (95:5) or air + CO_2 (95:5) was passed through them via the tubes while they were shaken gently. At the end of the equilibration with the gas phase (10 min.) samples (1 ml.) were rapidly removed for initial glucose and lactate determination and also for glucosazone preparation and specific-activity measurement (10 ml.). The flasks were then stoppered tightly with the serum caps in place and shaken at 35°. At the end of 3 or 6 hr. n-NaOH (1 ml.) was added from a syringe with a thin needle, via the serum cap, to the inner tube containing filter paper and the contents of the flask were acidified with 2*N*- H_2SO_4 (1 ml.) via the other capped tube. The flasks were shaken at an increased rate for 20–30 min. to aid absorption of the CO_2 , and at the end of this time the absorption tubes were removed, the traces of alkali on the serum-capped tubes were rinsed into them with a few millilitres of CO_2 -free water and then more CO_2 -free water was used to elute $\text{Na}_2^{14}\text{CO}_3$ from the paper. The absorption tubes were rubber-stoppered and left standing

for 12 hr. Samples of the incubation medium were withdrawn from the flasks for final glucose and lactate determination. The remainder of the flask contents was then used for preparation of lactate.

Preparation of glucosazone. General procedures for preparation of radioactive samples are described by Steinberg & Udenfriend (1957). The method for the preparation of glucosazone containing ^{14}C isotope was that of Feller, Strisower & Chaikoff (1950). A minimum of two recrystallizations of the glucosazone were generally carried out before constant specific radioactivity was achieved.

Preparation of $\text{Ba}^{14}\text{CO}_3$. The filter paper was removed from the CO_2 -absorption tube and together with CO_2 -free water was used to elute $\text{Na}_2^{14}\text{CO}_3$, placed in a flask and again washed with water. The supernatant was centrifuged in stoppered tubes to remove cellulose fibres and the $\text{Ba}^{14}\text{CO}_3$ precipitated by addition of BaCl_2 (12%, w/v) containing a small amount of NH_4Cl . The precipitate was washed carefully with water, transferred quantitatively to a watch glass and dried.

Isolation of labelled lactate. A measured volume (30 ml.) of the incubation medium was removed from the test flasks and about 100 mg., accurately weighed, of pure anhydrous zinc lactate added to each. After deproteinization with sodium tungstate (5%, w/v) and H_2SO_4 (0.6*N*) the supernatant was continuously extracted with warm ether for 24 hr., and the lactic acid was isolated according to the method of Bernstein & Wood (1957). The acid was then neutralized with zinc carbonate, boiled to destroy the inner lactone and, after evaporating to the point of crystallization in a stream of air, ethanol-acetone (90:10) was added until a faint turbidity appeared. On setting aside at 0° for a few hours crystals of zinc lactate formed. These were collected and recrystallized from ethanol-acetone. The salt was then dehydrated in an air oven at 110° and stored in a vacuum desiccator.

Isolation of labelled glycerol. Extraction of glycerol from the incubation medium by deproteinization and evaporation to dryness over P_2O_5 , followed by extraction with ethanol and chromatography, was carried out according to the method used by Grant & Fulton (1957). Ethanolic eluates from the chromatographic strips were pooled, concentrated to small bulk in a ground-glass stoppered tube, and the glycerol present was benzoylated by adding 0.5 ml. of 30% (w/v) NaOH , followed by cautious addition of a few drops of benzoyl chloride with shaking. After the reaction was complete the solid was washed on the filter with a large volume of water and the residue dried in a desiccator over CaCl_2 . In view of the small yield, no recrystallization was attempted.

Isolation of spirochaete material. After incubation the organisms were isolated by centrifuging, washed twice in 0.9% NaCl and freeze-dried. The dry material obtained was used for counting without further treatment.

Specific activity of samples. Measurements of radioactivity were made on 0.3 cm.² Perspex planchets that had been previously calibrated to obtain the factor for conversion into planchets of 1 cm.² area. Counts of the amounts of radioactivity in the different samples, whether BaCO_3 , glucosazone or zinc lactate (Karnovsky *et al.* 1955), were made on a Dynatron scaler with thin end-window Geiger-Müller counter, at infinite thickness, unless otherwise specified. In cases where samples were not infinitely thick correction factors were applied (Calvin, Heidelberger,

Reid, Tolbert & Yankwich, 1949). Correction of the lactic acid activity to allow for the added carrier was made by means of the equation given by Arnstein & Grant (1957). Where warranted, coincidence corrections for the particular counter used were made. The usual error associated with radioactive measurements of this kind arising out of variation in preparation of samples and statistical errors in counting was estimated to be $\pm 5\%$.

Reagents. General reagents were of AnalaR grade. The purest obtainable specimens of sugars were supplied by British Drug Houses Ltd. [$U-^{14}C$] and [$1-^{14}C$] Glucose were purchased from The Radiochemical Centre, Amer-sham, Bucks.

RESULTS

Metabolism of Spirochaeta recurrentis in laboratory rats

The suitability of a number of laboratory animals was tested for this strain of parasite normally maintained by serial passage in mice. Animals tested were the multimammate rat (*Mastomys coucha*), gerbils (*Meriones libicus*), hamsters (*Mesocricetus auratus*), guinea pigs and rats, and the most suitable was the albino rat, of weight 70–80 g. Twenty of these heavily infected animals provided sufficient material for experiment.

The disease in rats is rarely fatal, although their food and water consumption is reduced during infection. Leucocytosis is marked, the spleen and liver are enlarged and, when infections become heavy, bile pigments are present in the blood. The peak of infection is reached in 2 days with the inoculum used and falls to zero in a matter of hours without reappearance of parasites over a period of 6 weeks. During this time the rats cannot be reinfected. If the blood of animals from which the parasites had disappeared was added to spirochaetes, used for inoculation of fresh animals, marked clumping of the parasites took place and the resulting degree of parasitaemia was reduced.

Biochemical changes in the host during infection

Preliminary work on the chemical changes brought about in the rat by the spirochaete was undertaken to obtain information on the nature of the metabolism of the parasite. Blood-glucose concentrations were found to be decreased pro-

portionally to the degree of parasitaemia as with other blood parasites (Christophers & Fulton, 1938; Mercado, 1952). Detailed estimation of blood glucose and liver glycogen revealed that there was a fall in glucose and an even greater fall in liver glycogen (see Table 1), in spite of the fact that experiment showed that the host food intake was not seriously reduced.

Examination of the end products of metabolism in the host blood

The large demands made by the spirochaetes on the blood glucose of the rat host led to a search for α -keto acids, but only pyruvate was detectable. Lactic acid was also found to be present in large amounts. The determination of these two substances in the blood of normal and infected animals was therefore undertaken.

The normal value of pyruvate in rat blood is about 0.7–1.1 mg./100 ml. (Goodwin & Williams, 1952). In our normal host animals the value was 1.6 mg./100 ml. and this value was trebled in rats infected with *S. recurrentis* (Table 1). The level of lactic acid in the host blood rose suddenly until at the height of infection its value was also trebled. The lactic acid values fell rapidly as the parasites disappeared from the blood. In view of the large amount of acidic products present in the blood during infection the respiratory CO_2 output was measured. From the results shown in Table 1 it is apparent that rats expire significantly more CO_2 only at the peak of infection. In view of the above findings the investigation *in vitro* described below was undertaken.

Metabolism of Spirochaeta recurrentis in vitro

S. recurrentis is a fragile organism and when isolated from formed blood elements it was found that motility could best be preserved when cells were suspended in buffered medium–glucose at 35° with gentle agitation. The addition of albino rat serum up to 5% was beneficial, possibly by reason of its buffering capacity. In medium alone, with no energy source, aggregation readily occurred. Similarly, clumping was brought about by horse or rabbit serum. A useful index to the vitality of spirochaetes was provided by motility observed

Table 1. *Biochemical changes in the rat during laboratory infection with Spirochaeta recurrentis*

Average values and variations of the mean are given.								
Period of infection*	No. of rats	Av. wt. (g.)	Glucose (blood) (mg./100 ml.)	Glycogen (liver) (mg./100 ml.)	Liver wt. (g.)	Pyruvate (blood) (mg./100 ml.)	Lactate (blood) (mg./100 ml.)	Respiratory CO_2 (g./hr./100 g. rat)
Controls	23	85 $\pm$ 5	100.4 $\pm$ 2.2	4.96 $\pm$ 0.44	3.8 $\pm$ 0.3	1.60 $\pm$ 0.07	47.6 $\pm$ 2.3	0.456 $\pm$ 0.085
1	10	86 $\pm$ 4	—	—	—	—	75.6 $\pm$ 5.9	0.496 $\pm$ 0.047
2	23	76 $\pm$ 5	43.7 $\pm$ 3.5	1.51 $\pm$ 0.30	4.7 $\pm$ 0.2	4.58 $\pm$ 0.06	146.0 $\pm$ 10.6	0.771 $\pm$ 0.037
3	10	77 $\pm$ 3	—	—	—	—	61.0 $\pm$ 3.8	0.472 $\pm$ 0.034

* Figures indicate: 1, first day after infection; 2, at peak of infection; 3, day after peak of infection.

under dark-ground illumination. Since the parasites were observed to lose motility in the absence of glucose this method was used as a test for the specificity of various substrates (Fulton & Spooner, 1956).

Substrate specificity. The final suspension of organisms as isolated from the blood was diluted and washed two or three times with warm 0.9% NaCl until all were immobilized, care being taken not to keep them too long in the absence of sugar. The energy sources used and results obtained are given in Table 2.

It is clear that the chief substrates required by *S. recurrentis* are glucose and fructose. The changes in pH value indicate that with glucose an appreciable amount of acid is formed. Lactose, maltose and glycerol also appeared to support motility to a smaller extent. Neither galactose nor the pentoses were metabolized.

Gaseous exchanges. Preliminary experiments with *S. recurrentis* *in vitro* failed to show an uptake of O₂ or CO₂ production except when white blood cells had not been completely removed from the preparation. There was, however, a marked utilization of glucose to the extent of a half to three-quarters of the dry weight of the organisms/hr.

The evolution of CO₂ mentioned by earlier writers (Fenyvessy & Scheff, 1930; Scheff, 1935) remained to be explained. Experiments were therefore designed to measure gas exchanges in *S. recurrentis* under varying experimental conditions, with concomitant estimation of glucose used and lactic acid produced. These results are shown in Table 3. There is no apparent O₂ uptake or CO₂ output by the organisms themselves. The stoichiometric relations between glucose used and lactic acid formed show that the reactions were predominantly glycolytic in character.

Table 2. *Substrate specificity for maintenance of motility in Spirochaeta recurrentis*

Flasks (in duplicate) contained substrate (5 mm, 10 mm), immobilized spirochaetes (0.1–0.2 mg./ml. dry wt.) and 0.9% NaCl to a total volume of 5 ml. Blanks were run with no substrate. pH at start, 7.4. Temp. 35°. In air. Duration of expts. 1 hr.

Substrate	Activity*		ΔpH	
	5 mm	10 mm	5 mm	10 mm
D (+)-Glucose	+++	+++	-0.8	-1.2
D (-)-Fructose	++	+++	-0.7	-0.8
D (+)-Mannose	-	-	-0.1	-0.1
L (-)-Sorbitose	-	-	0.0	0.0
D (+)-Galactose	-	-	-0.1	0.0
-Lactose	+	+	-0.2	-0.2
-Maltose	+	+	-0.3	-0.3
D (+)-Ribose	-	-	-0.1	-0.1
D (+)-Arabinose	-	-	0.0	-0.1
-Glycerol	-	+	-0.2	-0.2

* Symbols indicate: -, no spirochaetes motile; +, a few spirochaetes alive but majority immobile; ++, half of total motile; +++, all vigorously motile.

Identification of end products of spirochaetal metabolism

Paper chromatography of the volatile and non-volatile organic acid fractions was carried out as described above. Only lactic acid was detected. By the more sensitive method of gas-phase chromatography, however, traces of *n*-butyric acid, isovaleric acid and *n*-valeric acid were found (Fig. 1). Pyruvic acid and traces of glycerol were also detected by the methods described. The presence of appropriate controls confirmed that these end products were the result of glucose catabolism.

In view of these findings an attempt was made to obtain a balance between glucose utilization and pyruvate and lactate production *in vitro* (Table 4). It appears that the utilization of glucose is accounted for almost completely by the lactic acid and pyruvic acid produced. The increase of pyruvate with time is thought to be due to accumulation of lactate causing reversal of the equilibrium

Table 3. *Origin of carbon dioxide in suspensions of Spirochaeta recurrentis*

Warburg flasks contained spirochaetes plus Krebs medium, or, in 30% (v/v) concentration, normal rat serum or rat serum which had been first acidified, evacuated and then neutralized, or whole rat blood. Energy source was glucose (0.3%). Blanks contained all the above-mentioned except spirochaetes. Centre wells of flasks measuring O₂ uptake contained 0.2 ml. of 20% KOH. Side arms of flasks measuring CO₂ output contained 0.5 ml. of 2N-H₂SO₄, which in some flasks was tipped before, and in others after, the experiment, to allow for CO₂ retention by the medium. Temp. 37°. Gas phase, air. Total liquid volume, 3.2 ml. Duration of expts. 1 hr.

Suspending medium	Total O ₂ uptake (μl.)		Total CO ₂ output (μl.)		Total glucose utilization (μmoles/ml.)	Total lactic acid production (μmoles/ml.)	Glucose:lactic acid ratio (molar)	Glucose converted into lactic acid (%)
	Spirochaetes	Blank	Spirochaetes	Blank				
Krebs medium	0	2	16	12	5.83	10.74	1:1.84	92
Serum	2	0	73	69	7.55	14.67	1:1.94	96
Treated serum	0	0	15	8	3.42	6.66	1:1.96	98
Whole blood	36	50	125	124	10.13	19.72	1:1.95	97

pyruvate $\rightleftharpoons$ lactate in favour of pyruvate production. The predominantly glycolytic nature of the reactions taking place is again apparent.

Acid production as a measure of glycolytic activity and inhibition by various reagents. When the spirochaetes were suspended in 0.9% NaCl containing 0.2% of glucose in the presence of 0.01M-sodium bicarbonate the CO_2 produced in the presence of different substrates could easily be measured. The results confirmed the substrate-specificity tests described in which glucose was found to be the primary energy source. The same conditions were used to measure inhibition of acid production, as described in Table 5. These conditions are closely similar to those used by Lardy & Phillips (1943*a, b*) and by Mann & Lutwak-Mann (1948) in their work on sperm glycolysis and respiration. The pH was approximately 7.0 when a gas phase of $\text{N}_2 + \text{CO}_2$ (95:5) was used as 37° (Umbreit *et al.* 1957). The inhibition by fluoride is reversible, a phenomenon first noted by Lardy & Phillips (1943*b*) during studies on sperm glycolysis. Iodoacetate inhibition

of acid production by spirochaetes was complete and irreversible. As would be expected in organisms without aerobic requirements the addition of azide to the suspension medium makes little or no difference to the metabolic rate. By far the most marked effect was obtained by pyruvate. This reagent is well known as a glycolytic stimulant in certain concentrations (Mendel, Bauch & Strelitz, 1931; Bumm, Appel & Couciero, 1933) and, in fact, at the smaller concentration used here a 50% stimulation of the rate of acid production occurred. The reactions were slowed when the pyruvate concentration was raised. The addition of adenosine triphosphate and Mg^{2+} ions failed to cause any enhancement of the rate of acid production, and likewise the addition of several sugar phosphate esters was without effect, owing possibly to permeability factors.

Experiments with radioactive glucose

The results shown in Table 6 provide additional proof that CO_2 is not given out during the metabolism of *S. recurrentis* *in vitro*. The very small evolution of CO_2 recorded is insignificant, being of the order of 0.2% of the total glucose utilized when allowance is made for blank experiments. The picture does not appear to be markedly different with either $[\text{U}-^{14}\text{C}]$ - or $[1-^{14}\text{C}]$ -glucose.

Table 7 shows as well that no incorporation of radioactive label occurs in either the spirochaetes themselves or in the small amount of glycerol formed under the conditions of the experiment.

DISCUSSION

It has been shown in these studies that *S. recurrentis* consumes large amounts of glucose *in vitro*. Since the glucose content of the host blood is decreased in proportion to the degree of parasitaemia, a fact first noted by Fenyvessy & Scheff (1930), it is not unreasonable to suppose that this substrate plays an important part in the metabolism of the parasite *in vivo*. Support for this view is provided by the fact that these rats were allowed food without restriction and intake was not significantly reduced except for a few hours at

Fig. 1. Gas-liquid partition chromatography of *S. recurrentis* volatile acids. Metabolic products (50 ml.) were neutralized and freeze-dried. The dry residue was taken up in water (5 ml.) and an equivalent volume of orthophosphoric acid (60%, w/v) added. The mixture was placed on a Celite- Na_2SO_4 (anhyd.) column, eluted with dry ether, evaporated and distilled on to an acid-washed Celite 545 column. Column length, 4 ft.; liquid phase stearic acid (10%, w/v) in DC 550 silicone; N_2 pressure, 47 cm. Hg; temp. 100°. A, Test sample; B, marker sample.

Table 4. Glucose, lactate and pyruvate balance in *Spirochaeta recurrentis* metabolism *in vitro*

Suspension (25 ml.) of *S. recurrentis* in medium + glucose (0.2%) was incubated at 35° on a shaker in air. Samples were withdrawn at zero time and at intervals during the course of incubation.

Time (min.)	Glucose utilized ($\mu\text{moles/mg. dry wt.}$)	Lactic acid formed ($\mu\text{moles/mg. dry wt.}$)	Pyruvic acid formed ($\mu\text{moles/mg. dry wt.}$)	Lactic acid (% glucose converted)	Pyruvic acid (% glucose converted)
30	8.7	16.8	0.8	93.0	4.6
60	16.7	30.0	2.0	90.0	6.0
120	23.7	46.0	3.6	97.0	7.6
180	25.5	47.8	4.4	94.0	8.7

Table 5. *Inhibition and stimulation of Spirochaeta recurrentis metabolism*

Warburg flasks contained 1 ml. each of spirochaete suspension, glucose (24 μ moles) and 0.01M-sodium bicarbonate in 0.9% NaCl. Side arms contained 0.5 ml. of solution of inhibitor or stimulator to give the indicated molarity. Duplicate samples of suspension, treated with 0.1M-potassium fluoride and incubated for 30 min., were washed three times with 0.2% glucose-0.9% NaCl to remove fluoride and set up similarly to controls. After equilibration flasks were tipped. Temp. 37°. Gas phase $N_2 + CO_2$ (95:5). Total vol. 3.5 ml.

Additions	Concn. (M)	Inhibition (-) or CO ₂ activation (+)	
		(μ l./hr./mg. dry wt.)	(%)
Fluoride	0.10	5 $\pm$ 3	97 (-)
Azide	0.001	165 $\pm$ 15	8.8 (+)
Iodoacetate	0.0001	0 $\pm$ 3	100 (-)
Pyruvate	0.10	38 $\pm$ 5	75 (-)
	0.001	234 $\pm$ 23	55 (+)
Control	—	151 $\pm$ 14	0
Washed	—	142 $\pm$ 8	6.6 (-)

the peak of infection. In malaria and trypanosome infections the liver-glycogen content is also decreased (Christophers & Fulton, 1938; Mercado & Brand, 1954). There is a marked increase in lactic acid and pyruvic acid in the blood, in agreement with the production of these substances *in vitro* by the parasite. Coleman & Brand (1957) have shown that during haemoprotozoan infections in the rat pyruvic acid concentrations in the blood are raised. The pronounced rise in the blood concentrations of lactic acid in spirochaete-infected rats doubtless throws some strain on the buffering capacity of the host blood and disposal of lactic acid as carbon dioxide and water seemed probable. We have confirmed this experimentally with carbon dioxide. The study of gaseous exchanges in trypanosome-infected rats by Brand (1951) has shown that a greater amount of carbon dioxide is given out by infected animals in the 36 hr. preceding death, when parasites become very numerous.

It seems strange that in an aerobic environment this parasite should not consume any oxygen. This finding is in agreement with that of Fenyvessy & Scheff (1930) later confirmed by Scheff (1935). McKee & Geiman (1950), using a North American strain of relapsing-fever spirochaete, reported that there was a small oxygen uptake by the parasite *in vitro*, but actual figures were not given. It is well known (Hindle, 1931) that a pronounced leucocytosis always occurs when the parasites are at the peak of infection. As leucocytes are known to have a high rate of aerobic respiration (Coxon & Robinson, 1956) it is possible that this could explain the oxygen consumption noted by these authors.

Results obtained in the experiments described in Table 3 show clearly that there is no carbon dioxide

production by the organisms themselves. The observation by earlier workers that carbon dioxide was produced by the organisms apparently was based on the fact that addition of fresh normal rat serum to the suspension caused increased gas production, adequate controls were lacking. From the stoichiometric relations given in Tables 3 and 4 it seems that the metabolic reactions of the spirochaetes are predominantly glycolytic in character. This requires a 1:2 molar relationship between glucose utilized and lactic acid produced, which in fact was found to be the case. The presence of a glycolytic pathway (or a minor variant or both; Entner & Doudoroff, 1952) is suggested by the results obtained with an inhibitor such as fluoride, which interferes with a phosphorylative type of metabolism (Claridge & Werkman, 1953). Further, the effect of iodoacetic acid in small concentration on enzymes containing essential sulphhydryl groups (Barron, 1951) selectively inhibits triose phosphate dehydrogenase (Yang, 1957).

In contrast with the relatively large amounts of lactic acid produced by *S. recurrentis*, *n*-butyric acid, valeric acid and isovaleric acid occurred only in amounts detectable by delicate techniques of gas-phase chromatography and may in the absence of acetic acid be of significance only in the amino acid or fat metabolism of the organism as coenzyme A derivatives (Rendina & Coon, 1957; Masoro, Felts, Panagos & Rapport, 1957).

The presence of a higher amount of radioactivity in the evolved carbon dioxide from breakdown of [$1^{14}C$]glucose, compared with that from the [$U^{14}C$] or [$6^{14}C$] species, is taken to indicate, with certain reservations, the operation of a shunt mechanism of the hexose monophosphate type (Wood, 1955; de Duve & Hers, 1957). This method of determining metabolic activity has been used by various workers (Bloom, Stetten & Stetten, 1953; Bloom & Stetten, 1953; Santer & Ajl, 1955; and Coxon & Robinson, 1956). The assumption made is that the carbon in position 1 of the 6-phosphogluconate molecule is split off as carbon dioxide under the action of 6-phosphogluconate dehydrogenase if the hexose monophosphate pathway is operative. Consequently, if all the radioactivity is centred at the C_1 site in the glucose any carbon dioxide liberated will contain all the label from this source. Further the recent assessment of the isotopic method for evaluation of the routes of glucose utilization in micro-organisms by Dawes & Holms (1958) emphasizes the weighting of the evidence in favour of the presence of a hexose monophosphate pathway by use of [$1^{14}C$]glucose. Therefore the finding that in *S. recurrentis* no $^{14}CO_2$ is evolved, even with glucose labelled at C_1 , is evidence strongly in favour of the non-existence of an oxidative cycle of carbohydrate metabolism.

Table 6. *Distribution of radioactivity from labelled glucose in vitro: distribution of ^{14}C among metabolic products*

Spirochaete suspensions (50 ml.; 100 ml. in the preliminary experiment) containing $[\text{U-}^{14}\text{C}]$ glucose (0.3%; approx. $0.75\ \mu\text{C}$) or $[1\text{-}^{14}\text{C}]$ glucose (0.3%, approx. $0.75\ \mu\text{C}$) were incubated at 35° under the conditions and for the times indicated. Glucose and lactate were estimated colorimetrically at the beginning and end of each experiment, and were counted as glucosazone and anhydrous zinc salt respectively. Carbon dioxide was absorbed in NaOH and counted as $\text{Ba}^{14}\text{CO}_3$. —, Signifies value not determined. Controls contained the same constituents and activity as described above but no spirochaetes.

Experimental conditions and label used	Duration (hr.)	Specific radioactivity ($\mu\text{C/g.}$)		Original activity (%)	Total radioactivity utilized or produced (counts/min.)		Total activity (%)
		Glucose	Lactic acid		Glucose	CO_2	
Preliminary air; $[\text{U-}^{14}\text{C}]$ glucose + Krebs medium alone	6	4.34	—	—	26 700	62	0.23
Aerobic; $[\text{U-}^{14}\text{C}]$ glucose	3	2.07	2.08	> 100	7 740	31	0.40
+ 5% serum $[1\text{-}^{14}\text{C}]$ glucose	3	1.97	1.96	99	7 630	38	0.49
Control; $[\text{U-}^{14}\text{C}]$ glucose	3	—	—	—	—	22	—
Anaerobic; $[\text{U-}^{14}\text{C}]$ glucose	3	2.04	1.94	95	6 925	25	0.36
+ 5% serum $[1\text{-}^{14}\text{C}]$ glucose	3	2.27	2.28	> 100	8 200	52	0.63
Control; $[\text{U-}^{14}\text{C}]$ glucose	3	—	—	—	—	17	—

Table 7. *Distribution of radioactivity from labelled glucose in vitro: distribution of ^{14}C among organisms and glycerol*

Experimental details were as given in Table 6. Spirochaetes were isolated and glycerol was extracted after incubation as described in the Methods and Materials section.

Experimental conditions and labelled glucose used	Duration (hr.)	Specific activity ($\mu\text{C/g.}$)			
		Glucose	BaCO_3	Spirochaetes	Glycerol
Anaerobic; $[\text{U-}^{14}\text{C}]$ glucose + 5% serum	6	1.475	0.000	0.014	0.120
Control; $[\text{U-}^{14}\text{C}]$ glucose	6	1.659	0.040	—	—

It must be concluded therefore that as the major portion of the ^{14}C label in glucose appears quantitatively in the lactic acid formed, without loss by way of carbon dioxide, anaerobic glycolysis by the Embden-Meyerhof route must be the only pathway of glucose catabolism present *in vitro* in *S. recurrentis*. In the light of preliminary enzyme experiments, reported later (Smith, 1960a) it seemed unlikely that *S. recurrentis* possesses enzymes of the Entner-Doudoroff pathway (Entner & Doudoroff, 1952) found in some bacteria (Gunsalus, Horecker & Wood, 1955), so that the overall picture provided by these studies is that the spirochaete of relapsing fever possesses a phosphorylative type of metabolism based on anaerobic glycolysis. The final proof of this belief is provided by the enzymic experiments described in the next papers (Smith, 1960a, b).

SUMMARY

1. The metabolism of *Spirochaeta recurrentis*, the parasite of relapsing fever, has been studied *in vitro* and *in vivo*.

2. Large quantities of lactic acid produced in

the blood of the rat host result in a raised output of respiratory carbon dioxide at the peak of infection which is accompanied by hypoglycaemia and depletion of liver glycogen.

3. Experiments *in vitro* have shown that there is no aerobic respiration and that the spirochaetes catabolize glucose by a glycolytic mechanism predominantly to lactic acid, which is accompanied by a small amount of pyruvate. In addition very small amounts of glycerol, *n*-butyric acid, normal and isovaleric acids are formed.

4. Inhibition studies of metabolism by fluoride, iodoacetate and azide and results obtained with $[\text{U-}^{14}\text{C}]$ - and $[1\text{-}^{14}\text{C}]$ -glucose have confirmed that metabolism of the organisms was of a glycolytic nature.

5. No carbon dioxide attributable to the organisms themselves was given out during catabolism *in vitro* of either of the labelled glucose samples.

6. No incorporation of radioactive label occurs in the isolated organisms after incubation with labelled substrate.

7. The Embden-Meyerhof route of anaerobic glycolysis was the only pathway of glucose catabolism *in vitro* detected.

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