There is therefore enough evidence to show that the minerals of the teeth are not static. The apparent stability of the minerals of the teeth may be due to a dynamic state in which the equilibrium is much more resistant to change than is the case in bone, although it must be remembered that bone has a much greater blood supply than teeth.

The enzyme now known as alkaline bone phosphatase was first observed by Robison & Soames (1924); it is present in both teeth and bone. In enamel, the enzyme does not actually occur within the ameloblast but outside in the stratum intermedium (Bevelander & Johnson, 1945; Morse & Greep, 1947*a*, *b*). On the other hand, phosphatase occurs actually within the odontoblast and the adjacent pulp during mineralization of the dentine (Bevelander & Johnson, 1946). Histochemical methods have revealed that the ameloblasts contain more phosphorus than do the odontoblasts (Hintzsche & Baumann, 1933). By the use of micro-incineration techniques Hampp (1940) showed that the mineral content of the ameloblasts differed considerably from that of the odontoblast. There is therefore some evidence to suggest that the ameloblasts and odontoblasts differ chemically and biochemically.

The influence of the high-sugar diet on the enamel, dentine and bone of the rat lends support to the view that the processes of mineralization in the three tissues are significantly different.

SUMMARY

1. The calcium and phosphorus content and the calcium/phosphorus ratios in the rat femur were the same whether the animals were raised on a stock diet of Lever cubes or a diet containing 67 % sucrose.

2. This is in contrast to the effect on the enamel and dentine of the incisor teeth of the same rats (Hartles, 1951), and makes it clear that the 'sugar' disturbance is confined to the teeth and does not include bone.

3. The correlation between the amounts of calcium and phosphorus in enamel, dentine and bone has been evaluated.

4. The findings support the view that the processes of mineralization in the three tissues are significantly different.

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Studies on the Metabolism of the Protozoa

1. METABOLISM OF THE PARASITIC FLAGELLATE, *TRYPANOSOMA LEWISI*

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Trypanosoma lewisi has its natural environment in the blood stream of the rat. In this medium, oxygen, glucose and carbon dioxide are readily available to meet the energy requirements of the flagellate. Biot, Biot & Richard (1911) first indicated the important part played in the life of *T. lewisi* by glucose, which according to Reiner, Smythe & Pedlow (1936) is metabolized with the production of acid. Searle & Reiner (1941) showed that under aerobic conditions, almost 50 % of this acid was acetic acid, and stated that carbon dioxide had a pronounced stimulatory effect on the respiration. Under anaerobic conditions, they found that very little glucose was used, unless pyruvate or carbon dioxide was also present. In this latter case, they demonstrated the fixation of carbon dioxide, with the production of succinic, lactic, pyruvic and acetic acids. Moulder (1948*a*, *b*) showed that, in the adult phase, *T. lewisi* was able to oxidize glucose more efficiently than in the reproductive phase, and that the trypanosome was also

capable of oxidizing other substrates. In the present study, some of these results are confirmed and extended. New observations are reported concerning the aerobic and anaerobic rates of glucose utilization by *T. lewisi*, and results described of a quantitative study of the various metabolic products. The aerobic metabolism of *T. lewisi* is shown to depend on the cytochrome system. Experiments are described on the action of a variety of inhibitors, including a light-reversible inhibition by carbon monoxide.

MATERIAL AND METHODS

Trypanosome suspensions. The strain of *T. lewisi* used was isolated in 1945 by Dr P. Tate of the Molteno Institute, Cambridge, from a wild stack rat at Grantchester, and has been maintained in hooded or albino laboratory rats ever since, intraperitoneal or subcutaneous inoculations being made at least every 2 weeks. The infestation reaches a maximum of 1–3 × 10^5 flagellates/μl. blood on the 6th day of infection, and then slowly declines. The organisms used in this investigation have been harvested at 10–12 days, at an age corresponding to the so-called 'adult' phase of Taliaferro. Infected rats (about 200 g. body weight) were anaesthetized with ether, tied down, and the left interior carotid artery dissected out. A section of this was clipped off, a glass cannula inserted into the artery under a dissecting microscope, and on removing the clip, the blood was collected in 5 ml. of a 0·9% solution of NaCl containing 1% sodium citrate. In this manner about 8·5 ml. blood could be obtained from a 200 g. animal, the process taking about 12 min. Pooled samples of blood were centrifuged for 10 min. at 4000 rev./min. and the supernatant discarded. Most of the trypanosomes separated as a white layer above the red blood cells. They were collected with a Pasteur pipette, along with some red cells. The remaining red cells were twice suspended in a 0·9% solution of NaCl containing 1% glucose (glucose-saline), centrifuged, and each time the upper trypanosome layer was collected. The resulting preparation contained most of the trypanosomes, but only about one-thirtieth of the original red cells. The material containing the trypanosomes was diluted with glucose-saline, and the remaining erythrocytes removed by means of an agglutinating antiserum to rat erythrocytes, obtained from rabbits treated by the method of Moulder (1948*a*). Sufficient of this antiserum was added to the trypanosome suspension to agglutinate all the red cells. The mixture was incubated at 37° for 15 min. and the agglutinated blood corpuscles removed by centrifugation for 1 min. at a low speed. The supernatant containing the trypanosomes was then centrifuged at a higher speed, the trypanosomes washed two or three times, and made up into a suspension with a N content of 0·5–1 mg. N/ml. The suspending medium was either a Ringer-bicarbonate solution containing 0·1 M-NaCl, 0·007 M-KCl, 0·002 M-$MgSO_4$ and 0·016 M-$NaHCO_3$, which in the presence of 5% CO_2 in the gas phase has a pH of 7·3; or a Ringer-phosphate solution containing 0·094 M-NaCl, 0·005 M-KCl, 0·001 M-$MgSO_4$ and 0·025 M-phosphate (added in the form of sodium phosphate buffer, pH 7·3). The preparations of *T. lewisi* obtained by the above method were free from both erythrocytes and leucocytes, but often contained small clusters of blood platelets. 10^9 Protozoal cells are equivalent to about 8·8 mg. material (dry wt.), or 1 mg. N. This quantity of material can usually be obtained from the blood of a single rat. The results of measurements on the metabolic activities of trypanosomes were based on the N content of the suspensions, and expressed as q_{O_2} and $q^{N_2}_{CO_2}$, i.e. as μl. gas/mg. N/hr.

Spectroscopic observations on cytochrome were made by means of a microscope fitted with a Zeiss microspectroscope ocular. Observations were made both at room temperature, and at low temperatures, as described by Keilin & Hartree (1949, 1950).

Metabolic experiments. These were carried out at 37° in Warburg manometers, in cups (volume 20–25 ml.) fitted with two side arms. Gas phases of 5% CO_2–95% O_2, 5% CO_2–95% N_2, or air were used. The effect of CO on respiration was studied at 20° in Barcroft differential manometers. The source of light used to reverse the inhibition caused by CO was a mercury vapour lamp. The decrease in bicarbonate concentration in the medium during glucose metabolism gave a measure of the total amount of acid produced. Initial and final estimations of bicarbonate in the medium were made by tipping in 0·2 ml. 2 N-H_2SO_4 from a side bulb, and measuring the CO_2 expelled. The difference between the decrease in bicarbonate concentration and the CO_2 actually liberated during the period of glucose metabolism, gives a measure of the CO_2 formed or fixed. The manometer contents were then neutralized with 0·2 ml. 2 N-NaOH, and samples taken for estimation of the various metabolites.

Estimation of metabolites. Glucose was estimated iodometrically by the method of Somogyi (1945) in $Ba(OH)_2$-$ZnSO_4$ filtrates, or when very small samples only were available, in neutralized trichloroacetic acid filtrates by the method of Folin & Malmros, as described by Umbreit, Burris & Stauffer (1945). The method was modified by using 0·5% gelatin as stabilizing colloid in place of 2% gum ghatti, the colour being measured after 30 min. Gelatin has a zeta-potential about one-twentieth that of the various gums, and was found to be much more efficient in preventing the precipitation of the Prussian blue. Using trichloroacetic acid filtrates, pyruvic acid was estimated colorimetrically by the method of Friedemann & Haugen (1943), lactic acid by the colorimetric method of Barker & Summerson, as described by Umbreit *et al.* (1945), and citric acid by the method of Speck, Moulder & Evans (1946). Formic acid was estimated colorimetrically by the method of Grant (1947) on a sample of a distillate from a tungstic acid filtrate. Acetic acid was estimated in another sample of this distillate, by redistilling it from HgO, and titrating the distillate as described by Buchanan, Sakami, Gurin & Wilson (1947). Ethanol was estimated by the bichromate method of Nicloux, le Breton & Doutcheff (1934). The aniline blue spot test of Feigl (1937) was used to test for oxalic acid. Succinic acid was estimated manometrically by means of a washed horse-heart preparation of succinic oxidase. A preliminary ether extraction was not used, because it was found that none of the other metabolites present gave an O_2 uptake with the enzyme preparation, and succinic acid recoveries were the same as after prolonged ether extraction.

RESULTS

Aerobic metabolism

Under aerobic conditions in the presence of utilizable substrate, the trypanosomes are highly motile. In the absence of substrate, the organisms quickly

become immotile, but the motility can be restored by the addition of glucose, provided that the period without substrate has not been too long. Anaerobically, in the presence of glucose, the trypanosomes show a lower motility than under aerobic conditions, but this motility can be restored to normal by the admission of air.

Respiratory activity. A suspension of washed trypanosomes, in Ringer-phosphate solution in air, respires at a low, but steady rate over a period of several hours, with a q_{O_2} of about 40. However, several substances, when added to a washed suspension of trypanosomes, are able to stimulate this respiration over tenfold, and to restore the motility of the flagellates. In the presence of glucose, which is the oxidizable substrate the trypanosomes find *in vivo*, they respire with a q_{O_2} of about 600. On this level, the respiration continues at a constant rate over a period of an hour or two (Fig. 1). Using air as

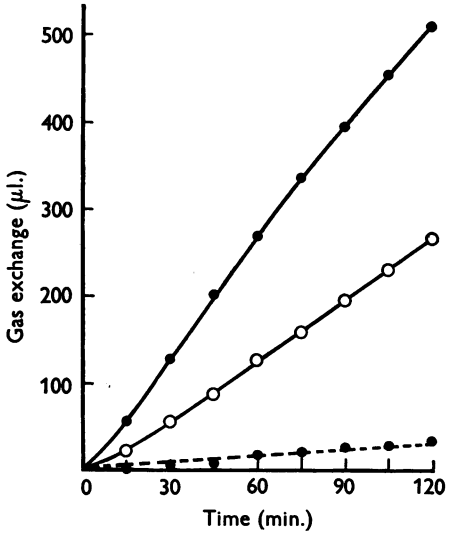

Fig. 1. Aerobic and anaerobic metabolism of glucose by *T. lewisi*. Each manometer cup contained 0·02 M-glucose and 0·48 mg. trypanosome N (approx. 5×10^8 cells) in a volume of 1·5 ml.; temp. 37°; ●——●, O_2 uptake (μl.) in presence of glucose; ● - - - - - - ●, O_2 uptake (μl.) in absence of glucose; ○——○, anaerobic CO_2 production (μl.) in bicarbonate buffer.

the gas phase, the ability of thirty-three different substances to stimulate respiration was tested. The substrate was added from a side bulb after the period of manometric equilibration to give a final concentration of 0·02 M, and respiration was followed over a period of 1 hr. or more. The substances examined fall into three groups:

(*a*) Glucose, mannose and fructose are readily oxidized with a q_{O_2} of about 600. Glutamine is oxidized with the liberation of free ammonia, with a q_{O_2} of 730.

(*b*) Glycerol is oxidized with a q_{O_2} of about 250, and asparagine and glutamic acid with a q_{O_2} of about 100.

(*c*) The following substances failed to increase respiration above the endogenous level, or support motility: galactose, sorbose, xylose; maltose, sucrose, lactose; mannitol, sorbitol, dulcitol, inositol; gluconic acid; DL-glyceraldehyde, dihydroxyacetone, pyruvate, lactate, ethylene glycol, ethanol; citrate, succinate, α-keto glutarate; formate, acetate, proprionate; glycine, alanine and aspartic acid. This list contains, among other substances, lactate and succinate, which are both known to be formed normally by *T. lewisi* during its anaerobic glucose metabolism. Additional evidence that these substances are not utilized aerobically by the protozoa, is shown by the following experiment. A suspension of washed trypanosomes was incubated anaerobically with glucose until all the sugar had been fermented. At this point, air was admitted to the incubated mixture. There was no significant oxygen uptake, nor was there any aerobic disappearance of lactic acid, which had accumulated during the anaerobic phase. In spite of the inability of lactate and succinate to increase the respiration, the trypanosomes are not devoid of the lactic and succinic dehydrogenases, as shown by experiments on homogenized cells (Table 1 and Fig. 4).

Table 1. *Succinic and lactic dehydrogenase activity in* Trypanosoma lewisi *homogenate*

(Homogenate prepared by shaking an aqueous suspension of trypanosomes with glass beads in Mickle disintegrator for 5 min. Phosphate buffer (pH 7·3) added to final concn. of 0·05 M. Thunberg tubes used, each containing 1·16 mg. trypanosome N, 0·03 M-substrate, and 0·134 μmol. methylene blue, added from a hollow stopper; total vol. 2·5 ml. Tubes evacuated and washed with pure N_2 six times. Temp. 37°.)

	Time for decolorization (min.)	
	90%	100%
Homogenate	>180	>180
Homogenate + succinate	37	51
Homogenate + lactate	42	62

Products of glucose oxidation. Using the indirect method of Warburg, determinations were made of the respiratory quotient and acid production during glucose oxidation. A manometer vessel of 13 ml. capacity, containing 5 ml. fluid, was used in conjunction with another of 23 ml. capacity, containing 1·2 ml. fluid. In each flask, the tissue/fluid and glucose/fluid ratios were the same. The organisms were suspended in a bicarbonate medium, and the carbon dioxide-oxygen gas phase was used. Initial and final acid tips were used to determine acid production, and initial and final glucose determinations were made by the iodometric method. Three

experiments gave R.Q. values of 0·94, 1·00 and 0·96 (mean 0·97), and showed that 1 mol. of glucose is oxidized by 3·17 mol. oxygen, to liberate 1·19 equiv. of acid and 3·06 mol. carbon dioxide. Using the direct method of Warburg with a phosphate buffer, the oxygen/glucose ratio (not corrected for the blank oxygen uptake) was 3·86 (an average of nine values, ranging from 3·51 to 4·18). An attempt was made to analyse completely the overall reaction occurring during glucose oxidation. The experiment was carried out in five manometers, the combined contents of which were analysed as indicated in Table 2. In the same table, a carbon and acid balance sheet for the oxidation is given.

Effect of inhibitors on respiration and motility. The sensitivity of respiration to a number of inhibitors was studied; the results are given in Table 3. In most cases the trypanosomes were allowed to respire in the main vessel of the manometer in the presence of 0·01 M-glucose for about 20 min. and then the inhibitor was tipped in from the side arm. In the case of cyanide, balanced potassium hydroxide-potassium cyanide mixtures were used in the centre well as recommended by Robbie (1946). 0·02 M-Fluoride had no observable effect on the motility of the trypanosomes, and only a small effect on respiration. 0·001 M-2:4-Dinitrophenol, which had approximately the same effect on respiration as 0·02 M-fluoride, reduced the motility practically to zero; motility was restored to normal on washing. 0·001 M-Cyanide rendered the trypanosomes almost immotile, but activity was restored if the hydrogen cyanide was removed. 0·0001 M-Iodoacetate, over a period of 45 min., gradually reduced the motility and respiration of the flagellates to zero; this effect was found to be irreversible. Carbon monoxide

Table 2. *Metabolic products formed during aerobic and anaerobic glucose breakdown*

(Each manometer flask contained 0·68 mg. trypanosome N, 4 mg. glucose added from side arm after equilibration, 0·016 M-bicarbonate; total vol. 3·0 ml.; temp. 37°; gas phase 5% CO_2–95% O_2 or 5% CO_2–95% N_2 (five flasks each). After 180 min. incubation, 0·4 ml. 2 N-H_2SO_4 added from second side bulb to stop reaction and to estimate residual bicarbonate. Contents of five flasks neutralized, pooled, and samples taken for estimation of the various metabolites. Yields expressed in terms of mol. metabolite found per mol. glucose disappearing, from which values for carbon and acid recoveries have been calculated.

During the aerobic reaction, a total of 81·7 μmol. glucose was used (by 3·4 mg. trypanosome N). Previous experiments had shown that for 1 mol. glucose oxidized, 3·17 mol. O_2 were used, and 3·06 mol. CO_2 were produced.

During the anaerobic reaction, a total of 72·2 μmol. glucose was used. For each mol. glucose used, a net fixation of 0·74 mol. CO_2 was observed.)

	Aerobically			Anaerobically		
Metabolite	Mol.	C atoms	Acid equiv.	Mol.	C atoms	Acid equiv.
Carbon dioxide	3·06	3·06	—	—	—	—
Succinic acid	0·18	0·72	0·36	0·89	3·56	1·78
Lactic acid	0·12	0·36	0·12	0·39	1·17	0·39
Pyruvic acid	0·03	0·09	0·03	0·19	0·57	0·19
Acetic acid	0·47	0·94	0·47	0·18	0·36	0·18
Ethanol	0·05	0·10	—	0·02	0·04	—
Formic acid	0·00	—	—	0·00	—	—
Oxalic acid	0·00	—	—	0·00	—	—
Citric acid	0·00	—	—	0·00	—	—
Sum	—	**5·27**	**0·98**	—	**5·70**	**2·54**
C atoms supplied (as glucose and CO_2)	—	6·00	—	—	6·74	—
Acid yield (from bicarbonate used)	—	—	1·19	—	—	2·50

Table 3. *The effect of various inhibitors on respiration*

(Each manometer vessel contained about 0·4 mg. trypanosome N in a Ringer-phosphate medium, pH 7·3. Glucose concentration 0·01 M. Total vol. 2·0 ml.; temp. 37°; gas phase air, with 0·2 ml. 20% KOH in centre well. O_2/glucose ratio represents the number of mol. O_2 utilized/mol. glucose disappearing. Figures in brackets represent the ratio in absence of inhibitor.)

Inhibitor	Concentration (M)	Inhibition (%)	O_2/glucose ratio
2:4-Dinitrophenol	0·001	24	—
Sodium fluoride	0·02	18	—
Sodium arsenite	0·0001	59	2·58 (3·54)
Potassium cyanide	0·00001	52	2·26 (4·05)
Sodium azide	0·001	27	3·13
Sodium azide	0·005	58	2·04
Hydroxylamine	0·0004	18	4·00 (4·13)
Hydroxylamine	0·002	56	2·48
Carbon monoxide	95% in gas phase	38	—

(95% in the gas phase) inhibited the respiration by 38%, and the effect was found to be reversible by light (Fig. 2). Disappearance of glucose in the presence of cyanide, azide, hydroxylamine and arsenite, was not inhibited to the same extent as oxygen uptake. A comparison of data in Tables 3 and 5, shows that the aerobic reactions are in general

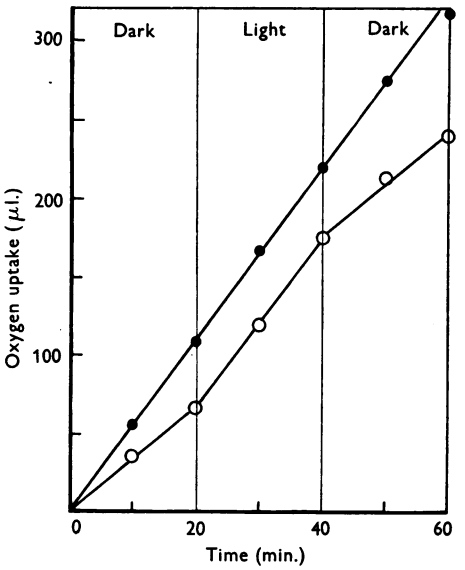

Fig. 2. Inhibition of respiration of *T. lewisi* by CO. Experiment carried out in Barcroft differential manometers at 20°; each cup contained 2·1 mg. trypanosome N and 0·02 M-glucose in 3 ml. Ringer-phosphate solution; 0·3 ml. 20% (w/v) KOH in centre well; ●——●, O_2 uptake (μl.) in presence of 5% O_2 and 95% N_2; ○——○, O_2 uptake (μl.) in presence of 5% O_2 and 95% carbon monoxide, during alternating 20 min. periods of darkness and light.

more sensitive to inhibitors than are the purely anaerobic ones. Oxygen/glucose ratios fall from about 3·8 in the control to 2·5 or less in the presence of inhibitor, indicating that under these conditions the anaerobic reaction is playing a more prominent part.

Anaerobic metabolism

Rate and pathway of anaerobic glucolysis. Values obtained of the $q_{CO_2}^{N_2}$ are rather variable. From seventeen different trypanosome preparations, values ranging from 190 to 470 were obtained, with an average of 360. The actual q value obtained depends to a large extent on the treatment of the organisms. The higher values were obtained when the trypanosomes were kept supplied with glucose during all stages in the preparation of the washed suspension; the lower ones when the flagellates were washed with glucose-free solutions and kept in such solutions during the period of manometric equilibration, prior to tipping in glucose from the side arm. Fig. 1 illustrates the progress of the anaerobic glucolysis.

Searle & Reiner (1941) state that under anaerobic conditions very little glucose is metabolized unless carbon dioxide or pyruvate is also present. The experiment described in Table 4 confirms these observations, showing that pyruvate increases glucose utilization threefold, while carbon dioxide increases it 12-fold. It is interesting to note that per mol. glucose utilized, more lactic acid is produced in the absence, than in the presence, of carbon dioxide. Table 4 also shows the relative rates of glucose utilization under aerobic and anaerobic conditions. As can be seen from the table, carbon dioxide causes only a very slight increase in glucose utilization, accompanied by a small decrease in the lactic acid yield.

Using five manometer vessels, a large-scale fermentation was carried out, to determine the end products of glucolysis. The results are given in Table 2, expressed in terms of mol. of metabolite formed per mol. glucose utilized. It can be seen from the table that these figures are fairly consistent from the point of view of carbon and acid balance. Yields of the various metabolites were variable; on some occasions, twice the yield of lactic acid has been observed, but this was accompanied by a corresponding decrease in the amount of carbon dioxide fixed and succinic acid formed. There was always a correlation between the lactic and succinic acid yields.

Table 4. *The effect of* CO_2, O_2 *and pyruvate on glucose metabolism*

(Each manometer flask contained 0·88 mg. trypanosome N, phosphate buffer pH 7·3 or 0·016 M-bicarbonate as appropriate, and 1 mg. glucose tipped in from side arm at zero time; total volume 1·6 ml. Reaction stopped after 100 min. by tipping in 0·2 ml. 2 N-H_2SO_4.)

System	Glucose used (μmol.)	Lactic acid formed (μmol.)	Ratio lactic acid/glucose
Glucose + N_2	0·39	0·57	1·45
Glucose + N_2 + 0·02 M-Pyruvate	1·36	2·08	1·52
Glucose + N_2 + 5% CO_2	4·99	2·03	0·41
Glucose + air (no CO_2)	5·10	0·85	0·17
Glucose + O_2 + 5% CO_2	5·23	0·58	0·11

Effect of various inhibitors on glucolysis. Experiments were carried out with a number of inhibitors at different levels of concentration. The inhibitor was neutralized where necessary, and made up in a solution containing 0·016M-bicarbonate and 0·03M-glucose. Sodium chloride was added to bring the total cationic concentration up to 0·125M. Manometers were used containing 1 ml. trypanosome suspension in the main vessel, and 0·5 ml. of the solution containing glucose and the inhibitor in the side arm. This was tipped in after 10 min. equilibration, and the evolution of carbon dioxide was followed. Table 5 gives, in most cases, the inhibitor concentration found necessary to reduce glucolytic carbon dioxide output by 50 %. It can be seen from Table 5 that the anaerobic glucose metabolism is very sensitive to 2:2′-dipyridyl and 8-hydroxyquinoline. The effect of both these metal-binding substances develops immediately upon their addition to glycolysing organisms; in the presence of inhibitor, acid production continues at a steady, but much reduced rate. The anaerobic metabolism is also quite sensitive to iodoacetate, arsenite, cyanide and fluoride, but it is not inhibited by malonate or phenylthiourea. In view of the large anaerobic production of succinate, malonate was tested as an inhibitor; concentrations up to 0·04M were without effect on the rate of acid production.

Table 5. *The effect of various inhibitors on anaerobic glucose metabolism*

(Manometer cups contained about 0·6 mg. trypanosome N, 0·01M-glucose, bicarbonate buffer and 5 % CO_2–95 % N_2; total vol. 1·5 ml.; temp. 37°; pH 7·3. Glucose metabolism measured in terms of CO_2 liberated from bicarbonate.)

	Concentration (M)	Inhibition (%)
Potassium cyanide	0·007	50
Sodium azide	0·025	50
2:2′-Dipyridyl	0·0003	72
8-Hydroxyquinoline	0·0003	78
Phenylthiourea	0·001	0
Sodium fluoride	0·004	50
Sodium iodoacetate	0·0001	50
Sodium malonate	0·04	0
Phlorrhizin	0·01	50
Sodium arsenite	0·003	50

Using the concentrations of inhibitors necessary to reduce the glycolytic carbon dioxide output by about 50 %, the metabolic products of glycolysis were examined in a number of cases, to see if the yields differed in any way from the control. Manometric experiments were carried out at the same time, with and without inhibitor. The reaction was stopped by tipping in 0·4 ml. 2N-sulphuric acid from a side bulb. After measuring the residual carbon dioxide liberated, 0·4 ml. 2N-sodium hydroxide was added. Succinic acid was determined in a 1 ml. sample, and glucose, lactic and pyruvic acids in another 1 ml. sample, deproteinized with 10 % trichloroacetic acid. With azide or cyanide, the overall glucose utilization was reduced, but the amounts of succinic, lactic and pyruvic acids formed per mol. glucose used were not changed. With 0·005M-fluoride, glucose utilization was reduced, and although results were rather variable, there was a definite increase in the ratios of carbon dioxide fixed and succinic acid formed, with a corresponding decrease in the yields of pyruvic and lactic acids.

The cytochrome system and cytochrome oxidase

Spectroscopic evidence. A thick trypanosome suspension was made in glucose-Ringer-phosphate solution. Examination of this suspension showed that the cytochrome was mostly in the reduced form. Reduction was completed by adding sodium dithionite ($Na_2S_2O_4$), and the suspension was examined spectroscopically at normal and at low temperatures. In the latter case, warm glycerol was added to the trypanosome suspension to give a concentration of about 50 %. A little sodium dithionite was added to reduce the cytochromes, and the mixture was introduced into a long Pyrex tube 12 mm. in diameter, the end of which had been blown to a flattened disk-shaped cell, about 25 mm. in diameter, with an optical depth of 4 mm. The tube was quickly cooled in liquid oxygen, the mixture solidifying to a transparent glass. Spectroscopic examination showed that the cytochrome bands were sharpened and displaced towards the blue, but rather weak. As the tube warmed up, the colourless vitrified mass acquired a transient amber hue, which faded as devitrification of the solid mass took place. Crystallization was accompanied by a marked intensification of the bands. The microcrystalline mass was then cooled again in liquid oxygen. Spectroscopic examination showed that the cytochrome bands were sharpened and considerably intensified, revealing a more detailed structure, and supporting the observations made at normal temperatures. At room temperature the spectrum showed:

(*a*) A definite, but very weak cytochrome *c* band at 550 mμ.

(*b*) A band extending from 553 to 560 mμ., with a definite maximum at 559 mμ., and a possible slight reinforcement at 553 mμ. This band may represent one component *e* or modified *b*, or may be due to two components, a weak *e* band at 553 mμ., and a strong *b* band at 559 mμ.

(*c*) A band at 605 mμ., representing cytochrome *a*.

(*d*) Corresponding β-bands in the region 525–535 mμ.

Manometric evidence. A homogenate of *T. lewisi* was prepared by shaking an aqueous suspension of the organisms with glass beads in the Mickle disintegrator for a few minutes. Using the system of

Keilin & Hartree (1947), it was shown that this homogenate could oxidize *p*-phenylenediamine, and that the oxidation was stimulated by added cytochrome *c* (Fig. 3). The trypanosome homogenate was also able to oxidize succinic acid, this oxidation being stimulated by added cytochrome *c* (Fig. 4). By using a small amount (0·5 mg.) of succinic acid, and allowing the oxidation to go to completion, it was shown that one atom of oxygen was used for each molecule of succinic acid oxidized. In connexion with the presence of cytochrome oxidase, the inhibitory effects of cyanide and carbon monoxide on respiration described in Table 3 and Fig. 2 should be noted.

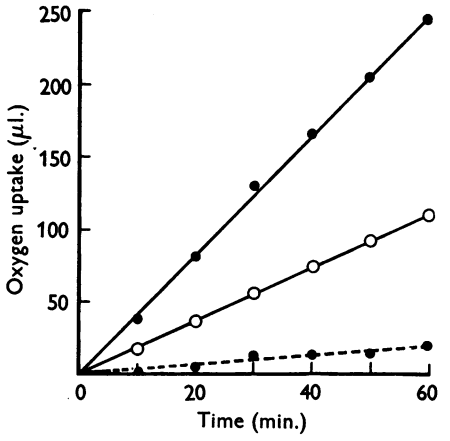

Fig. 3. Cytochrome oxidase activity in *T. lewisi* homogenate. Manometer cups contained trypanosome homogenate corresponding to about 5 mg. nitrogen, 13 mg. *p*-phenylenediamine tipped in from side arm after manometric equilibration, 0·3 ml. of 0·6% cytochrome *c* solution tipped in from second side arm, 1 ml. 0·1 M-phosphate buffer (pH 7·3), and KOH in centre well; gas phase air; temp. 37°; ●——●, O_2 uptake (μl.) of homogenate in presence of *p*-phenylenediamine and cytochrome *c*; ○——○, O_2 uptake (μl.) of homogenate in presence of *p*-phenylenediamine; ● - - - - - - ●, O_2 uptake (μl.) of *p*-phenylenediamine and cytochrome *c* in the absence of homogenate.

DISCUSSION

In rat blood, which provides the natural environment for *T. lewisi*, the survival and normal high metabolic rate of the trypanosomes depend largely on blood glucose as the source of nutrient material. A similar close dependence on glucose is shown by the trypanosomes after they have been separated from blood, and transferred into artificial, isotonic media. A general survey of the carbohydrate requirements and metabolism of trypanosomes has been recently given by von Brand (1950).

Aerobically, in absence of glucose, a suspension of washed trypanosomes in Ringer-phosphate solution, containing 10^9 protozoal cells, and representing about 9 mg. in dry weight, or about 1 mg. cellular nitrogen, takes up no more than 40 μl. oxygen/hr. at 37°. Presumably this low level of respiration is maintained at the expense of an intracellular nutrient reserve of the protozoal cells. As yet, however, there is little information on the chemical nature of this intracellular nutrient. It has been impossible to detect glycogen in the cells of *T. lewisi*, but this should not be regarded as a general characteristic of the Protozoa. In a recent, as yet unpublished study of another protozoan, *Glaucoma piriformis*, intracellular glycogen contents as high as 22% (on a dry weight basis) have been observed.

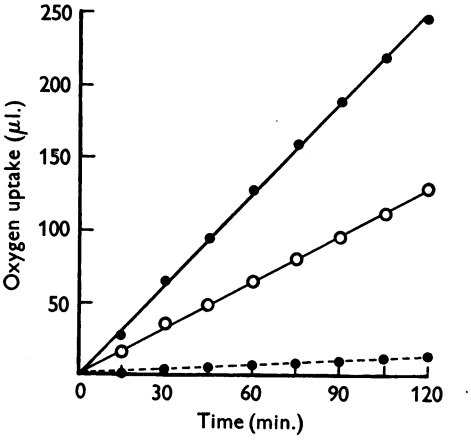

Fig. 4. Succinic oxidase activity in *T. lewisi* homogenate. Manometer cups contained trypanosome homogenate corresponding to 0·7 mg. nitrogen, in phosphate buffer (pH 7·3); 0·4 ml. of 0·2 M-succinate and 0·3 ml. of 0·6% cytochrome *c* solution tipped in from side arms after manometric equilibration; total volume 2·3 ml.; temp. 37°; gas phase air, with KOH in centre well; ●——●, O_2 uptake (μl.) of homogenate in presence of succinate and cytochrome *c*; ○——○, O_2 uptake (μl.) of homogenate in presence of succinate alone; ● - - - - - - ●, O_2 uptake (μl.) of homogenate alone.

On addition of glucose to a washed suspension of *Trypanosoma lewisi*, the rate of oxygen uptake immediately rises from 40 to 600 μl. oxygen/mg. nitrogen/hr., and remains at this high level for several hours. At the same time, the addition of glucose markedly enhances the motility of the trypanosomes. Fructose and mannose increase the oxygen uptake to the same extent as glucose, but galactose, sorbose, xylose, sucrose, maltose or lactose cause no increase. Glutamine stimulates the oxygen uptake of *T. lewisi* even more markedly than glucose, and a definite, though much smaller, increase has been observed with glycerol, asparagine and glutamic acid.

Glucose is oxidized by *T. lewisi* with an R.Q. of 0·97, and for each molecule of glucose oxidized, 3–4 mol. of oxygen are consumed, and about 1 mol.

equiv. of organic acid produced. The yield of aerobically produced acid, as revealed by the present study, is somewhat smaller than that reported by Searle & Reiner (1941), but in agreement with these authors, it was found that close to 50 % of the aerobically formed acid is derived from acetic acid. The rest is mainly composed of lactic and succinic acids. It is interesting to note that neither acetic, lactic nor succinic acids seem to be oxidized further by the intact cells themselves. This, however, does not preclude the existence in the trypanosome of intracellular enzymic systems active towards these organic acids. In cell homogenates it was possible to demonstrate the presence of lactic and succinic dehydrogenases, cozymase, and a complete succinic-oxidase system. Commenting on the fact that pyruvate, succinate, fumarate and α-ketoglutarate were not oxidized at significant rates by *T. lewisi*, Moulder (1948*a*) remarked that 'it is unlikely that the tricarboxylic acid cycle of Krebs is active in these organisms'. Analysis of the overall reaction of glucose oxidation by *T. lewisi* suggests a mechanism similar to that in the malarial parasite (Speck *et al.* 1946). With this organism, glucose is first converted to pyruvate, part of which is oxidized to acetic acid, the rest being completely oxidized by the tricarboxylic acid cycle. It was possible to demonstrate the stimulatory effects of various organic acids and co-factors only after liberation of the plasmodia from the red blood cells; it seems that the actual parasite is much more permeable to polar molecules, than is the host erythrocyte.

The present study also reveals the existence of a complete cytochrome system in *T. lewisi*. The close dependence of the respiratory activity of the trypanosome on the cytochrome system is further demonstrated by the light-reversible inhibition of the respiration by carbon monoxide. Cyanide ($4{\cdot}6 \times 10^{-4}$ M) completely blocks the respiration, and also renders the cells immotile. Dinitrophenol (10^{-3} M), on the other hand, causes almost complete inhibition of motility but only a negligible inhibition of respiration. This is presumably due to the known 'uncoupling' action of this compound on processes of oxidative phosphorylation. These results were obtained at pH 7·3, a rather high pH for the action of dinitrophenol; it is likely that the uncoupling effect of this compound would have been more pronounced at pH 6, but the trypanosomes do not respire well at this pH. Respiration is 59 % inhibited by 0·0001 M-arsenite, a value in close agreement with that found by von Brand, Tobie & Mehlman (1950). This sensitivity of respiration to arsenite is only about one-quarter of that observed with the African pathogenic trypanosomes. The effects of azide on both respiration and anaerobic glucose metabolism are interesting; usually the inhibitory effects of this substance follow closely those of cyanide. The fact that azide was found to have a much smaller effect than cyanide is probably explained by the high pH used (7·3).

Anaerobically, the trypanosomes are unable to survive unless extracellular carbohydrate is provided. In the presence of glucose under optimal conditions, the $q^{N_2}_{CO_2}$ may reach a figure as high as 470. For each molecule of glucose fermented anaerobically, approximately 2·5 mol. equivalents of organic acid are formed, composed mainly of succinic and lactic acids, with small amounts only of pyruvic and acetic acids. The anaerobic metabolism of *T. lewisi* was found to be extremely sensitive to a low concentration of 2:2′-dipyridyl or 8-hydroxy-quinoline, but not to phenylthiourea. From this it would seem likely that fermentation is dependent on the presence of an unco-ordinated heavy metal, quite probably ferrous iron, but not copper.

The initial stages of glucose breakdown by *T. lewisi* have not yet been elucidated. A number of trypanosomes, which are unable to ferment or oxidize glucose beyond the pyruvate stage, have been shown to carry out phosphorylative glycolysis (Chen & Geiling, 1946; Marshall, 1948; Harvey, 1949). It would seem likely, therefore, on a comparative basis, that *T. lewisi* breaks down glucose in a similar manner. The inhibitory effect of phlorrhizin on the fermentation also seems to indicate the participation of phosphorylative enzymes. However, attempts to demonstrate hexokinase activity in lysates or homogenates of *T. lewisi* have so far been unsuccessful. Direct oxidation of the glucose molecule seems a most unlikely mechanism in view of the results obtained with azide and hydroxylamine. Assuming that this type of mechanism was present, the first step in glucose breakdown would be an oxidation of glucose to gluconic acid and hydrogen peroxide, such as occurs in the acid-producing mould fungi. The peroxide would then be utilized in further oxidative reactions by the mediation of catalase. Were the catalase in such a system to be completely inhibited, glucose oxidation would lead to an accumulation of peroxide, and reduce the oxygen/glucose ratio to unity. As shown, however, in the present study (see Table 3), 0·001 M-azide or 0·0004 M-hydroxylamine, concentrations far above those necessary for total inhibition of catalase, give only small inhibitions of respiration and have very little effect on the oxygen/glucose ratio. Direct evidence of the occurrence of a phosphorylative mechanism of carbohydrate metabolism in *T. lewisi* still remains to be established.

SUMMARY

1. The motility and metabolism of *Trypanosoma lewisi* can be maintained *in vitro* both aerobically and anaerobically in the presence of suitable extracellular nutrients.

2. The respiration of *T. lewisi* depends on the cytochrome system. It is very sensitive to cyanide. It is also inhibited by carbon monoxide, this inhibition being light-reversible.

3. In the absence of extracellular nutrients, *T. lewisi* takes up oxygen at a rate of 40 μl. oxygen/mg. cellular nitrogen (or about 10^9 cells) in 1 hr. at 37°. In the presence of added glucose, mannose, fructose or glutamine, the q_{O_2} value increases from 40 to 600 or more. Glycerol, asparagine and glutamic acid increase the oxygen uptake to a smaller extent.

4. Glucose is oxidized by the trypanosome with an R.Q. of 0·97. For each molecule of glucose utilized aerobically, 3–4 mol. oxygen are taken up, and 1 mol. equivalent of organic acid is produced; half of this is acetic acid, and the rest mainly succinic and lactic acids.

5. The substances which are not utilized by the trypanosome aerobically include: galactose, sorbose, xylose, sucrose, lactose, maltose, lactate, pyruvate, succinate, citrate, α-ketoglutarate, acetate, formate, glycine, alanine and aspartic acid. Added lactate and succinate are not oxidized by intact cells, but lactic and succinic dehydrogenases could be demonstrated in cell homogenates.

6. Anaerobically, *T. lewisi* ferments glucose, the fermentation being stimulated threefold by 0·02M-pyruvate, and 12-fold by 0·016M-bicarbonate. The maximum value of the fermentation rate observed in the presence of carbon dioxide was $q^{N_2}_{CO_2} = 470$. For each mol. of glucose utilized anaerobically, 0·74 mol. carbon dioxide are fixed, and 2·5 mol. equivalents of organic acid are produced. The bulk of the acid accumulating anaerobically is succinic acid (70%) and lactic acid (15%); the rest is pyruvic and acetic acid.

7. The anaerobic fermentation is very sensitive to metal-binding substances, being inhibited by about 75% by 0·0003M-2:2′-dipyridyl and 8-hydroxy quinoline.

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The Dimensions of the Particle of Hyaluronic Acid Complex in Synovial Fluid

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A hyaluronic acid complex was separated from ox synovial fluid by Ogston & Stanier (1950) by ultrafiltration. This complex contained about 30% protein, and was considered to be the form in which hyaluronic acid occurs in the native fluid; removal of the protein led to degradation. The authors concluded, from their results on the sedimentation and diffusion of this complex, that the mean particle weight is 1×10^6 and the axial ratio about 60. Their values for the intrinsic viscosity indicated an axial ratio of nearly 600, and they interpreted this discrepancy in terms of interaction of the particles. This interpretation is logically unsound, because all the values used had been extrapolated to zero