The Metabolism of *Leishmania donovani* in Culture

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Leishmania donovani, the organism causing visceral leishmaniasis, is a small, round or ovoid body measuring 2–5 μ . in diameter, which occurs exclusively within cells of the reticulo-endothelial system of its mammalian host. In culture, or in the insect vector, the parasite exists in a flagellate leptomonad form. In the case of *L. tropica* in culture, it was shown by Salle & Schmidt (1928) that glucose was utilized, and that volatile acids and ammonia appeared in the culture medium. Reports on the metabolism of sugars in cultures have also been made by Kligler (1925–26), Noguchi (1926), Senekjic (1939), and Senekjic & Zebouni (1940). As serum was present as a component of the medium, the presence of glucose must be assumed. Wider metabolic studies on the flagellates were made by Adler & Ashbel (1934, 1940), Chang & Negherbon (1947), Chang (1948), and Fulton & Joyner (1949). The last-named authors compared the differences between the *in vitro* metabolism of the flagellate and tissue stages of *L. donovani*. The influence on oxygen uptake of various substrates and types of inhibitor including effective chemotherapeutic agents was measured, and respiratory quotients were determined. In the present investigation, the acid products formed during metabolism have been isolated, and the quantitative formation of these and of certain keto compounds was studied.

MATERIALS AND METHODS

The strain of *L. donovani* used was that previously described (Fulton & Joyner, 1949), and had been kept by serial passage in hamsters since 1939. Flagellate forms of the parasite were readily obtained by aseptic culture of infected liver or spleen. The composition of the culture medium and the method of obtaining large quantities of flagellates for respiration and other studies have already been described by these authors. The medium consisted of salts, glucose, rabbit serum, liver extract, peptone, and haemoglobin solution. Analysis showed that the final concentration of glucose was between 2100 and 2240 μ g./ml., and the pH between 8.0 and 8.2. In order to follow the production of various substances in culture, a large number of flasks containing the liquid medium were given the same inoculum and grown at 25° under identical conditions. The required number of flasks was then used at prescribed intervals for purposes of estimating the quantity of metabolic products present, the

period of maximum growth under the conditions employed having been shown to occur between the 10th and 14th days after inoculation. Control flasks containing culture medium alone were always used simultaneously with the experimental flasks. Since it was not possible to estimate all metabolic products in one sample of culture material, the data given in Table 1 for these substances are not inter-dependent.

Identification of metabolic products. In order to identify the acid products of metabolism, the flagellates from cultures of different ages were removed by centrifuging and the medium was deproteinized by adding H_2SO_4 and Na_2WO_4 and centrifuging again. The deproteinized filtrate was kept at –2° until sufficient material had been accumulated for chemical investigation. Several litres of metabolic solution were concentrated by distillation under reduced pressure at a temperature below 40° until about one-fiftieth of the original volume of metabolic liquid remained; suitable traps were used to retain any volatile acids. The total distillate was brought to pH 7.2 with dilute NaOH, and the salts formed were evaporated to dryness on a steam bath. The residue from distillation was then exhaustively extracted with ether, and, on removal of the solvent, crystalline and amorphous, viscous materials were obtained. Following a suggestion of Dr A. J. P. Martin, F.R.S., and Dr A. T. James, it was found possible to effect complete separation of the substances present by use of a column of dimensions 75 × 4 cm. and packed with Hyflo Super-cel previously mixed with half its weight of 0.5N- H_2SO_4 , which mixture had been equilibrated with ether and kept wet with the latter during the preparation of the column. Chemical investigation had shown that the substances present in the ether extract were lactic and succinic acids, and a trial run with these substances was made on the column by adding them in a small volume of ether and then eluting with the same solvent. A collecting siphon of about 5 ml. capacity was used, and titration of fractions with ethanolic KOH (approximately 0.1N) was carried out using bromothymol blue as indicator; by this means a titration curve could be constructed. A similar curve was also constructed for a mixture containing a large number of other organic acids which might occur as products of metabolism.

Quantitative analysis of metabolic products. At first, keto acids were estimated by the method of Lu (1939), which was not specific, and then by that of Friedemann & Haugen (1943), but the latter method gave anomalous results since we found that the amount of pyruvic acid (method A of these authors) slightly exceeded that of total keto acids (method B). Paper chromatography showed (Altmann, Crook & Datta, 1951) that this discrepancy could be traced to the presence of acetoacetate. When this substance is estimated by the methods of Friedemann & Haugen (1943), the values in terms of pyruvic acid are greater by their method A than by method B, with the result that pyruvic acid in presence of

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acetoacetate, other keto acids being absent, gives a higher value by the former method than by the latter. Attempts were then made to use the specific, yeast-carboxylase method (Umbreit, Burris & Stauffer, 1945) for estimation of pyruvic acid; this proved entirely satisfactory when sufficient material was available, but was not sensitive enough to estimate the amounts of pyruvic acid present in the culture medium of this investigation. At first we used as standard a specimen of ethyl pyruvate, which is readily hydrolysed, but later found that a pure specimen of the sodium salt was more satisfactory (Lipmann, 1944).

We used the method of Greenberg & Lester (1944) for the estimation of acetoacetate and acetone. In the early estimations the 2:4-dinitrophenylhydrazine was dissolved in HCl, but this salt gave consistently high blanks, and we finally used 5% (w/v) metaphosphoric acid as solvent for the base, which was satisfactory. Our standard was prepared by the procedure of Ljunggren (1924).

Succinic acid was estimated by the method of Krebs (1937), using a succinoxidase preparation from pigeon breast. The method described by Barker & Summerson (1941) was used to estimate lactic acid, for which a pure preparation of zinc lactate served as standard. That of Buffa & Peters (1949) was found convenient for the colorimetric estimation of citric acid.

The method of McLendon (1944) with the modifications of Phillipson (1947) was used in the estimation of volatile acids. In applying the method, three 30 ml. fractions were collected and titrated with 0.01 N (CO_2 -free), NaOH using an Agla micrometer syringe and, as indicator, 5 to 6 drops of 0.02% (w/v) phenol red containing 1 drop of 1/1000 methylene blue/5 ml. phenol red. The third fraction served as a blank for fractions 1 and 2. This indicator was apple green in the presence of acid, and, after passing through a colourless stage, changed to violet at pH 7.8. The methods described by James & Martin (1952) for the estimation of volatile acids when only small quantities of material were available were also kindly applied by them to our samples.

Glucose was estimated by the method of Miller & Van Slyke (1936). For isolation and recognition of those stable metabolic products which were formed in sufficient quantity, the methods described by Fulton & Stevens (1945) were used. Total acid production in the medium was followed by each day titrating a known volume of control medium with 0.01 N-HCl to bring the pH to that of the experimental flask. A growth curve was constructed by making haemocytometer counts of the number of flagellates present in a number of cultures of different ages. Measurements of oxygen uptake were made in an unmodified Barcroft apparatus.

RESULTS

Ethanol, acetaldehyde, and glycerol were not detected in the metabolic solution. By means of the column described, it was found that formic, α -oxoglutaric, oxalacetic, malic, malonic, maleic, and fumaric acids were absent. From the volatile fraction obtained by distillation under reduced pressure, acetic acid was isolated as the *p*-phenylphenacyl ester (m.p. and mixed m.p. 111°; prepared as described by Drake & Bronitsky, 1930). (Found: C, 75.4; H, 5.4. Calc. for $\text{C}_{16}\text{H}_{14}\text{O}_3$: C, 75.6; H, 5.5%.) When the substances present in the volatile

fractions were estimated by the elegant method of James & Martin (1952), the following relative amounts of acids were found to be present:

	Formic	Acetic	Propionic
Control (duplicates)	1.2, 0.8	5.8, 3.0	1.0, 1.0
Experimental	0	73.0	2.5

indicating that only acetic acid was formed in significant amounts during metabolism. In the ether extract of the residue from distillation, the crystalline material which was present in considerable quantity was shown to be succinic acid (by m.p., and m.p. mixed with a pure specimen, 185°). (Found: C, 40.5; H, 5.1. Calc. for $\text{C}_4\text{H}_6\text{O}_4$: C, 40.7; H, 5.1%.) Traces of this substance were also present in the control medium. The viscous residue from which the succinic acid had been separated was shown to contain lactic acid by converting it first into the zinc salt, then into the sodium salt, and finally into the *p*-phenylphenacyl ester (m.p. and mixed m.p. 145°). (Found: C, 71.4; H, 5.7%. Calc. for $\text{C}_{17}\text{H}_{16}\text{O}_4$: C, 71.8; H, 5.6%.) Lactic acid was also present in the control flasks. Pyruvic acid, which in previous experiments could not be isolated, was now obtained as the 2:4-dinitrophenylhydrazone, identical in m.p. and mixed m.p. with an authentic specimen (213°); the substance was not analysed. The acids present were estimated at intervals during the course of growth in different batches of flasks as shown in Table 1.

It will be seen from this table that there is a gradual fall in the pH of the medium as the culture ages, owing to the production of a number of acids. The rate of oxygen uptake decreased with the age of the cultures. In separate experiments, it was also found that glucose is more slowly metabolized by older organisms. Similar amounts of lactate were found both in the experimental and in the control flasks. It is possible that this was formed as an intermediate product. On the other hand, the amount of pyruvic acid present, as estimated by two different methods, steadily increased. The discrepancies which were noted in the results of the estimations of pyruvic and of total keto acid may be explained by the fact that two different methods of estimation were used, one of which, that for pyruvic acid, was influenced more by the presence of acetoacetate than was the other. An attempt to estimate pyruvic acid in the presence of acetoacetate, using aniline citrate to destroy the latter, gave unsatisfactory results. The presence of acetoacetate in small amounts in control and experimental flasks was demonstrated by paper chromatography and by colorimetric estimation. Free acetone was generally absent from the medium, but was detected in one culture to the extent of 4 $\mu\text{g./ml.}$ of medium on the thirteenth day. Citric acid was detected only in minute amounts. The chief product of metabolism

Table 1. *Quantitative formation of acid products during growth of Leishmania donovani in culture*

(Results expressed in μ moles./ml. of medium except where stated otherwise. E = Experimental medium. C = Control medium.)

Age of culture (days) ...	6	7	8	9	10	11	12	13	14
Total acid production	0.86	—	2.8	4.6	5.2	—	6.1	6.9	7.6
pH of medium	E 8.2 C 8.5	—	7.25 8.6	6.7 8.1	6.5 8.4	—	6.3 8.6	5.9 8.7	5.8 8.3
O ₂ uptake (in cu.mm./10 ⁸ parasites)	41.25	36.84	30.9	—	15.04	—	—	—	—
Glucose utilized	0.83	—	2.61	3.34	3.22	—	4.90	4.34	4.78
Acetic acid	E 1.85 C 0.05	—	1.73 0.085	2.06 0.17	2.13 0.30	—	1.92 0.24	—	—
Lactate	E 3.71 C 2.57	2.48 2.54	2.64 2.58	2.26 2.22	2.06 2.08	2.32 2.39	2.37 2.49	2.41 2.22	2.29 2.44
Pyruvate (method A of Friedemann & Haugen, 1943)	E 0.368 C 0.09	—	0.89 0.098	—	—	1.40 0.099	—	2.57 0.17	—
Acetoacetate	E — C —	—	—	0.236 0.263	—	0.254 0.230	—	0.290 0.260	—
Succinate	E Nil C Nil	Trace Nil	0.39 Nil	1.59 Nil	—	1.92 Nil	—	2.40 Nil	2.55 Nil

Fig. 1. The growth of *L. donovani* flagellates in cultures at 25°.

appears to be succinic acid, as was found to be the case for *Trypanosoma rhodesiense* (Fulton & Stevens, 1945). Acetic acid was the only volatile acid formed in significant amounts. When this substance was used in manometric experiments as a substrate, however, it caused only a slight increase in oxygen uptake by the flagellates of *Leishmania donovani*. The growth curve of *L. donovani* flagellates in culture at 25° is shown in Fig. 1.

DISCUSSION

L. donovani is among the few parasitic protozoa of which one developmental stage at least can be maintained in pure culture in a liquid medium. For this reason it lends itself to metabolic studies. The media

that are required, however, are complex, since serum and tissue extracts are essential constituents, and their presence proved disadvantageous when the products of metabolism were being isolated and their origins sought.

The importance of glucose as the main carbohydrate substrate of *L. donovani* has been previously demonstrated (Fulton & Joyner, 1949). The oxygen consumption and the activity of the flagellates were shown to depend on the presence of this sugar when all other organic material was removed. In the medium which was used, glucose was the main carbohydrate present, and its disappearance from the medium during the growth and multiplication of the flagellates was accompanied by the production of acids. The most rapid fall in pH corresponded with the presence of maximum numbers of dividing cells.

Of the products of metabolism, carbon dioxide, acetic, pyruvic, and succinic acids have been definitely identified. It is possible that lactic acid was formed as an intermediate product without accumulating in the medium. In addition to the above, Chang (1948) detected, without giving details, small quantities of formic acid in cultures after 1 week's growth, but we have been unable to confirm this by our methods. von Brand (1951) has summarized the end products formed during aerobic metabolism of certain of the Trypanosomidae, including *Leishmania*, and various trypanosomes found by different authors. The substances included pyruvic, lactic, formic, acetic, oxalic, and succinic acids, ethanol and glycerol.

In previous studies (Fulton & Joyner, 1949), it was shown that the respiratory activity of the flagellates was not constant; oxygen and glucose being consumed most rapidly during the earlier cultural stages. There was also a slight but definite fluctuation in the respiratory quotient of flagellates

of different ages, a peak value occurring on the eighth day. Our quantitative results also suggest that the metabolic mechanism may not be the same throughout the whole of the period of growth, since it was found that most of the acetic acid appeared during the first 6 days, while succinic acid was most actively formed during the later stages.

The metabolic pathways by which these substances are produced, and the differences between the metabolism of the flagellate and that of the intracellular stages of the parasite, must remain conjectural until more information is available. In particular, it would be interesting to know the part played by phosphorylation reactions and carbon dioxide fixation, and also the relationship between carbohydrate and protein metabolism which has been claimed by Salle & Schmidt (1928). Our findings, however, indicate the formation of several substances, the presence of which is not inconsistent with metabolic cycles known to occur in some other cells.

SUMMARY

1. A study of the biochemical activities of *Leishmania donovani* has been made *in vitro*.

2. During the period of growth glucose disappears from the culture medium, the pH of which falls in a manner related to the growth of the organisms.

3. Carbon dioxide, acetic, pyruvic, succinic, and probably lactic acids were produced, and their quantitative production during the growth of cultures was followed.

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Tricresyl Phosphates and Cholinesterase

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It has been stated that tri-*o*-cresyl phosphate (TOCP) is an inhibitor of cholinesterase *in vitro* (Hottiger & Bloch, 1943; Bloch, 1943; Earl & Thompson, 1952*a*). As a general rule, however, such stable organophosphorus compounds are poor inhibitors. Amongst a series of related compounds, a relationship between stability to hydrolysis and inhibitory power has been demonstrated (Aldridge & Davison, 1952*a, b*). Since TOCP is a stable substance, it was considered unlikely that it would have the inhibitory activity attributed to it, and this view was reinforced by the demonstration by Myers & Mendel (1953) that the inhibitory activity

of TOCP against the hydrolysis of tributyrin by rat serum varied considerably from sample to sample. The experiments described in this paper show that most of the inhibitory activity of at least one specimen of TOCP is due to an impurity.

TOCP has the interesting property of causing weakness and ataxia in the legs of chickens due to the demyelination of certain tracts of the spinal cord (Smith & Lillie, 1931). After feeding TOCP to chickens, a profound lowering of the pseudo cholinesterase at various sites is always obtained (Earl & Thompson, 1952*b*). Using a purified specimen of TOCP possessing little *in vitro* inhibitory activity,