CCLXXII. STUDIES IN THE METABOLISM OF PROTOZOA.

II. SOME BIOCHEMICAL REACTIONS OCCURRING IN THE PRESENCE OF THE WASHED CELLS OF *GLAUCOMA PYRIFORMIS*.

BY NORMAN RISBOROUGH LAWRIE.¹

From the Bonnett Memorial Laboratory, Addenbrooke's Hospital, Cambridge.

(Received August 1st, 1935.)

It is the purpose of the present communication to draw attention to the biochemical activity exhibited in a simplified environment by the protozoon *Glaucoma pyriformis*. The valuable data obtained by the investigation of the biochemical properties of suspensions of washed bacteria suggested the extension of such methods to the study of the protozoa. Such work is highly desirable on account of the biological and medical importance of this phylum. The remarkable environmental changes undergone by certain parasitic species of protozoa in the course of their life-cycle may be expected to furnish instructive instances of biochemical adaptations. A suitably chosen protozoal culture offers material for biochemical study which possesses a degree of homogeneity and freedom from specialisation of cellular function greater than that which obtains in the tissues of the higher animals. The complexity of the media required for the cultivation of the protozoa has hitherto rendered such work almost impossible. Within recent years, however, progress in the cultivation of protozoa in bacteriologically sterile, and even non-particulate, media, has rendered a limited number of species amenable to biochemical studies of this type [Lwoff, 1932].

Little biochemical work appears to have been carried out upon suspensions of protozoa separated from the medium upon which they have been grown. Hartog and Dixon [1893] collected specimens of the very large multinucleated protozoon *Pelomyxa palustris*. After treatment with alcohol, the protozoa were removed individually from contaminating material by means of a mounted needle, dried over sulphuric acid and pulverised. The resulting powder was moistened with alcohol and extracted with water. This extract readily hydrolysed starch to erythrodextrin (though the subsequent formation of reducing sugars was very slow) and dissolved fibrin in the presence of dilute acids. No change was perceptible when milk preserved with thymol was treated with the extract. Mouton [1902] described the preparation of a culture on gelatin containing one species of amoeba and one species of bacterium. A proteolytic enzyme in aqueous solution was obtained from these cultures. It was shown by control experiments that this enzyme could not have been extracted from the bacterial component of the cultures (*B. coli communis*). This protease, which was most active in slightly alkaline media, digested gelatin and fibrin but had little action upon coagulated egg albumin. Mesnil and Mouton [1903] cultivated *Paramoecium aurelia* in the presence of bacteria. The paramoecia, concentrated electrically, were able to liquefy gelatin. The reaction proceeded most rapidly in media which were neutral to litmus. Fenyvessy and Reiner [1928] measured the

¹ Beit Memorial Research Fellow.

respiration of trypanosomes washed by centrifuging and suspended in a solution of glucose and salts. Reiner and Smythe [1933-34] obtained suspensions of *Trypanosoma equiperdum* by fractional centrifuging of the blood of infected rats. Such suspensions, which contained very few red blood corpuscles, were shown to catalyse the breakdown of glucose into glycerol and pyruvic acid and the oxidation of glycerol to pyruvic acid.

The strain of *G. pyriformis* used in the present experiments was very kindly given to me by Dr Muriel Robertson, of the Lister Institute, London. It was cultivated upon a simple medium consisting of 1% "Difco" peptone and 0.4% of a simple salt mixture, dissolved in distilled water and sterilised by autoclaving. The bacteriological sterility of the cultures was tested from time to time by attempted aerobic subculture at 35° in glucose broth. The cultures used in the experiments described below were all bacteriologically sterile as judged by this test. Treatment of the culture with sterile quinamil (B.D.H.) solution was carried out as a precaution against bacterial contamination during the inoculations of numerous subcultures.

The cultures were allowed to grow for about 7 days. The protozoa were then separated from the culture fluid by centrifuging and twice washed by resuspension in 20 ml. of distilled water and centrifuging. The washed protozoa were then suspended in a suitably buffered solution, or in water, according to the requirements of the experiment in hand. The numbers of protozoa in the cultures were counted by means of the Fuchs-Rosenthal counting chamber. A considerable number of protozoa became disintegrated during these processes, so that the final reacting system contained not only intact cells, but also the insoluble products of cytolysis of some protozoa and any insoluble materials derived from either protozoa or medium during the development of the culture.

In order to test the suitability of these suspensions for biochemical study they have been examined for the presence of a representative series of enzyme systems, with the results described below.

PROTEASE.

Digestion mixtures containing suspension of *Glaucoma pyriformis*, buffer, protein solution and chloroform were set up in test-tubes and incubated, usually for 24 hours. Chloroform was used to cytolyse the protozoa and to inhibit bacterial growth and for these purposes was considered to be better than toluene.

The extent to which proteolysis had occurred was measured by precipitation of the undigested protein with trichloroacetic acid, filtration and estimation of the total nitrogen in a portion of the filtrate. For this estimation the method of incineration of Wong [1923] was found to be suitable, followed by distillation of the ammonia into standard acid and back-titration with carbon dioxide-free *N*/500 NaOH. This procedure permitted estimation of the non-protein-nitrogen to the nearest 1 mg. per 100 ml. of digest.

Boiled suspensions of *G. pyriformis* had no measurable proteolytic action on caseinogen. Autolysis at *p*_H 6.0 yielded only a small and variable increase in the non-protein-nitrogen of the digest, ranging from nil to 2 mg. per 100 ml. The following experiments are typical of the results obtained.

Exp. 1. Each digestion tube contained:

2% sodium caseinogenate	...	1 ml.
<i>M</i> /20 buffer	...	1 ml.
<i>Glaucoma</i> suspension	...	0.5 ml.
Chloroform	...	0.5 ml.

The population of the culture was such that if no loss of cells had occurred during centrifuging the digests would have contained 15.6×10^6 cells per 100 ml. The increase in non-protein-nitrogen after 24 hours' incubation at 30° is shown in Table I.

Table I. *Proteolysis of caseinogen in acid media.*

p_H	Increase in non-protein-nitrogen mg./100 ml.
2.2 phthalate buffer	5
4.0 " "	10
6.0 " "	16
6.0 phosphate "	21
7.0 " "	15
6.0 autolysis in phthalate buffer	2

The caseinogen was mostly out of solution in the tubes at p_H 4.0 and 2.2.

Exp. 2. A set of digestions was prepared similar to that used in Exp. 1. There was the equivalent of 32×10^6 cells present per 100 ml. of the digest. The results of 24 hours' incubation at 30° are shown in Table II.

Table II. *Proteolysis of caseinogen in alkaline media.*

p_H	Increase in non-protein-nitrogen mg./100 ml.
8.4 borate buffer	10
8.6 " "	9
8.8 " "	9
9.0 " "	8
9.2 " "	8
9.4 " "	7
9.6 " "	7

From these and similar experiments it was concluded that the optimum p_H for the proteolysis of caseinogen by a suspension of *G. pyriformis* was near 6.

In other experiments the sodium caseinogenate was replaced by egg albumin (B.D.H.). Very little proteolysis occurred, however, either with untreated or with heat-coagulated egg albumin. This difference in the ability of the *Glaucoma* suspension to proteolyse caseinogen and egg albumin respectively is brought out by the following experiment.

Exp. 3. To each of three test-tubes were added:

M/20 phosphate buffer at p_H 6.0	...	...	1.75 ml.
<i>Glaucoma</i> suspension	...	...	0.50 ml.
Chloroform	...	...	0.50 ml.

Table III. *Proteolysis of caseinogen and egg albumin compared.*

Tube	Increase in non-protein-nitrogen mg./100 ml.
A	3
B	16
C	2
	Increase in non-protein-nitrogen due to proteolysis of added substrate
A	1
B	14

To tube A were added 2.25 ml. of 0.5 % egg albumin, to tube B 2.25 ml. of 0.5 % sodium caseinogenate and to tube C 2.25 ml. of distilled water. These tubes contained the equivalent of 31×10^6 cells per 100 ml. The non-protein-nitrogen was estimated in each tube and they were then incubated for 24 hours. At the end of that period the non-protein-nitrogen was again estimated, with the results shown in Table III.

Thus it is clear that a suspension of *Glaucoma pyriformis*, saturated with chloroform, can carry out proteolysis at p_H values ranging from 2.2 to 9.6.

DIASTASE.

The presence of a diastase in suspensions of *G. pyriformis* is readily demonstrable. The following typical experiment shows this property. 11 ml. of a suspension of *G. pyriformis* in *M/20* phosphate buffer at p_H 7.0 were prepared and found to contain 34×10^6 cells per 100 ml. 5 ml. of this suspension were measured into two monax test-tubes, A and B. Tube A was placed in a boiling water-bath for 15 min. and then cooled. Both tubes then received 0.25 ml. of 1 % starch in 5 % sodium chloride solution and a few drops of toluene. They were then incubated at 37°. About 0.5 ml. was removed from each tube at intervals and tested for the presence of starch by means of a dilute iodine solution. The results obtained are shown in Table IV.

Table IV. *Diastatic action of G. pyriformis.*

	Result of iodine test for starch	
	Tube A	Tube B
At start of incubation	Deep blue	Deep blue
After 3 hours' incubation	"	Faint red
" 4½ "	"	"

In other experiments of a similar type but without the addition of toluene no colour was developed with iodine after 4 hours' incubation. These digests did not reduce Fehling's solution.

PEROXIDASE.

Preliminary experiments with the suspension of *G. pyriformis* showed that it exhibited strong peroxidase-like activity in the presence of 50 % acetic acid.

In order to determine whether a true peroxidase enzyme were present, or whether the effect were due to catalysis by haematin compounds, the following comparative experiments were carried out.

A suspension in water containing 52×10^6 protozoal cells per 100 ml. was prepared. Scrapings from horse-radish were ground with distilled water and the extract decanted off to provide a typical plant peroxidase system. A very dilute suspension of human blood in distilled water was used to provide a "pseudo-peroxidase" system. These three preparations were compared in the following series of experiments.

In Exp. I, 15 mg. of benzidine, 1 ml. of *M/20* phosphate buffer at p_H 7.0, 0.1 ml. of 20 vol. hydrogen peroxide and 0.25 ml. of enzyme preparation were mixed in a porcelain crucible. Exp. II was set up in the same way as Exp. I, except that the phosphate buffer was replaced by 50 % acetic acid. Exps. III and IV were repetitions of I and II respectively, in which the fresh catalytic systems were replaced by the same systems after they had been heated for 1½ hours in the boiling water-bath. The results of these experiments are summarised in Table V.

Table V. *The peroxidase action of a suspension of G. pyriformis.*

The sign + indicates a positive colour reaction and the sign - that no coloured product was formed.

Exp. no.	Source of catalyst		
	Horse-radish	Blood	<i>Glaucoma</i>
I } Unheated catalysts	+	-	-
II }	+	+	+
III } Heated catalysts	-	-	-
IV }	-	+	-

Exps. III and IV show definitely that the peroxidase in *Glaucoma* is thermolabile, and in this property differs from the peroxidase-like system of certain bacteria [Callow, 1926] and from the peroxidase-like action of haematin compounds.

When benzidine is used as the indicator of peroxidase action the activity of the peroxidase in the suspension of *Glaucoma* is greatest in acid preparations. In later experiments, using *p*-phenylenediamine, this peroxidase brought about oxidation at p_H 7.0, p_H 2.2, or in the presence of approximately 50 % acetic acid.

DEHYDROGENASE.

Suspensions of *Glaucoma* were tested for dehydrogenase activity by means of the Thunberg tube technique. Small tubes of about 5 ml. capacity were used, and contained:

Suspension	...	...	...	...	0.5 ml.
Buffered methylene blue solution	...	...	...	...	0.5 ml.
Substrate	...	...	...	...	0.5 ml.

There was a final concentration of methylene blue in the Thunberg tube of 0.005 % and of phosphate buffer at p_H 7.0 of *M*/60. The tubes were evacuated on the water-pump and placed in a water-bath at 37°. The time taken for complete decoloration was observed. It was found that the washed protozoal cells reduced the methylene blue rapidly in the absence of any added substrate. The rate of reduction of methylene blue was however accelerated by the addition of succinate to the system. Aeration, and starvation of the organisms in sterile distilled water, did not increase the acceleration of the rate of reduction of methylene blue caused by the addition of succinate. The results of a typical experiment are given in Table VI.

Table VI. *Reduction of methylene blue in the presence of G. pyriformis.*

The activity of the reducing system is expressed as 100/reduction time in minutes.

Substance added	Time required for complete reduction of methylene blue min.	Activity of reducing system	Increase in activity due to added substance
Water	28	3.6	—
<i>M</i> /15 sodium succinate	22½	4.4	25 %
<i>M</i> /15 glucose	28	3.6	Nil

Glucose and sodium succinate in the presence of boiled protozoal suspension did not reduce methylene blue. In addition to glucose, negative results were obtained with fructose, lactose, sodium formate and hypoxanthine. The increase in the activity of the reducing system caused by the addition of succinate varied

in different experiments from 25 to 43 %. It is concluded that succinic dehydrogenase is present in the cells.

Attempts to remove substrate from the cells by the methods of washing and aeration usually successful with bacteria only resulted in loss of activity of the enzyme. Treatment of the suspensions with toluene or phenol along the lines of the experiments of Quastel and Wooldridge [1927] upon the dehydrogenases of *B. coli* resulted in complete loss of dehydrogenase activity. The protozoon *G. pyriformis* differs from many bacteria and many animal tissues in the tenacity with which it retains substances capable of reducing methylene blue. Its behaviour in this respect resembles that of *Bacterium faecalis alkaligenes*, described by Quastel and Wooldridge [1925].

THE PRESENCE OF GLUTATHIONE IN *G. PYRIFORMIS*.

The nitroprusside test for the organic sulphhydryl group was applied to suspensions of *G. pyriformis* after the manner adopted by Callow and Robinson [1925] in the case of certain bacteria. 300 mrl. of a well-grown culture were centrifuged and the cells twice washed with 40 ml. of distilled water. The deposit of protozoa was then transferred to a small weighed dish and dried *in vacuo* over sulphuric acid to constant weight. 74 mg. of dry material were obtained. There were 34×10^6 cells in the material taken. 2 ml. of a saturated aqueous solution of ammonium sulphate were added to this dry material, well stirred and allowed to stand for 15 min. A few mg. of potassium cyanide were then added and the material filtered through a small plug of absorbent wool. To this filtrate 0.2 ml. of a freshly prepared 1 % aqueous solution of sodium nitroprusside and 0.2 ml. of ammonia solution (1 vol. of ammonia solution of sp. gr. 0.88 diluted to 5 vol. with distilled water) were added. A strongly positive reaction was given immediately. Subsequent similar experiments in which the addition of potassium cyanide was omitted also gave a positive reaction. 16.5 mg. of the dried protozoa were extracted with 2 ml. of 5 % trichloroacetic acid and tested for cystine by means of Sullivan's test. No cystine (or cysteine) was found to be present. It is concluded from these experiments that the cells of *G. pyriformis* contain glutathione in the reduced form.

SUMMARY.

The presence of a protease, diastase, dehydrogenase and peroxidase has been demonstrated in the protozoon *Glaucoma pyriformis*. The presence of glutathione in these organisms is inferred from the results of applying the nitroprusside test for the organic —SH group and Sullivan's test for cystine and cysteine to extracts of the dried cells.

I am deeply indebted to Sir F. G. Hopkins for his interest in this work.

REFERENCES.

- Callow (1926). *Biochem. J.* **20**, 247.
— and Robinson (1925). *Biochem. J.* **19**, 19.
Fenyvessy and Reiner (1928). *Biochem. Z.* **202**, 75.
Hartog and Dixon (1893). *Rep. Brit. Assoc.* 801.
Lwoff (1932). *Recherches biochimiques sur la nutrition des protozoaires.* (Masson et Cie, Paris.)
Mesnil and Mouton (1903). *Compt. Rend. Soc. Biol.* **13**, 1016.
Mouton (1902). *Ann. Inst. Past.* **16**, 457.
Quastel and Wooldridge (1925). *Biochem. J.* **19**, 652.
— (1927). *Biochem. J.* **21**, 148, 1224.
Reiner and Smythe (1933–34). *Proc. Soc. Exp. Biol. Med.* **31**, 1086.
Wong (1923). *J. Biol. Chem.* **55**, 431.