Brit. J. Pharmacol. (1959), **14**, 68.

STUDIES OF A PROTEOLYTIC ENZYME FROM *SCHISTOSOMA MANSONI*

BY

A. R. TIMMS* AND E. BUEDING

From the Departments of Pharmacology, Western Reserve University School of Medicine, Cleveland, and Louisiana State University School of Medicine, New Orleans, U.S.A.

(RECEIVED OCTOBER 20, 1958)

The presence of a proteolytic enzyme has been demonstrated in ground-up preparations of *Schistosoma mansoni*. A twenty-fold purification has been achieved by ultracentrifugation at *p*H 3.0; the enzyme has an optimum *p*H of 3.9 and a marked substrate specificity for haemoglobin. No significant proteolysis was observed either with whole serum at *p*H values of 3.9, 6.0 or 8.0, or with isolated serum proteins at *p*H ranges between 2.5 and 7.7. The evidence is discussed that this enzyme may be located in the intestine of the schistosomes and that it is, at least in part, responsible for the supply of amino acids to the organisms.

Little is known about the protein metabolism of *Schistosoma mansoni*. Rogers (1940) reported that the black pigment present in the intestine of *S. mansoni* is probably haematin formed by the digestion of haemoglobin from the host. The parasite must obtain nutrient materials, including amino acids, from the blood of the host, and it is conceivable that there is a mechanism for the degradation of one or more blood proteins, following ingestion into the intestine of the parasite. Halawani, Hafez, Newsome, and Cooper (1949) observed that haemoglobin disappeared from the medium in which *S. mansoni* survived *in vitro*.

If it were known precisely what amino acids are required by *S. mansoni* and by what mechanism the parasite obtains them, an opportunity might become available for the development of schistosomicidal drugs. An initial approach to this problem is reported in this paper, in which the demonstration, partial purification and properties of a proteolytic enzyme from *S. mansoni* are described. The enzyme shows a marked specificity for haemoglobin (Timms and Bueding, 1958).

METHODS AND MATERIALS

Survival Studies.—Survival of schistosomes in a synthetic medium was studied by the use of procedures reported previously (Ross and Bueding, 1950). The synthetic medium used in the present investigation contained the following constituents in 100 ml.: NaCl, 550 mg.; KCl, 1.25 mg.; Na_2HPO_4, 2.9 mg.; $NaHCO_3$, 20 mg.; glucose, 200 mg.; $CaCl_2$, 13.5 mg.; $MgCl_2$, 13.5 mg.; glutathione, 10 mg.; choline chloride, 0.2 mg.; calcium pantothenate, 0.2 mg.; ascorbic acid, 0.1 mg.; pyridoxine, 0.1 mg.; inositol, 0.1 mg.; niacin, 0.05 mg.; riboflavin, 0.02 mg.; thiamine, 0.01 mg.; carotene, 0.01 mg.; biotin, 0.005 mg.; folic acid, 0.005 mg. Tris (hydroxymethyl) aminomethane was used as a buffer in a final concentration of 0.0025 M. Before use, the *p*H of the medium was adusted to 8.0 with dilute NaOH. The composition of this medium, which represents a modification of the synthetic medium used previously, was arrived at by systematic changes in the concentrations of individual constituents. With a supplement of amino acids (see below), the worms survived from 2 to 9 days (Table I). Even in the absence of amino acids the worms remained alive for 1 to 3 days, while in the medium used in earlier experiments the worms died within 12 to 18 hr. (Ross and Bueding, 1950). Addition of purified fractions of protogen to this medium resulted in a marked increase in the survival of schistosomes (Ross and Bueding, 1950). Since these observations were made, protogen has been obtained in pure form and identified as α-lipoic acid (Brockman, Stockstad, Patterson, Pierce, Macchi, and Day, 1952; Reed, Soper, Schnakenberg, Kern, Boaz, and Gunsalus, 1952). Pure samples of α-lipoic acid, kindly supplied by Dr. T. H. Jukes and by Dr. I. C. Gunsalus, did not prolong the survival of schistosomes in this synthetic medium. Therefore, the observed effects of protogen fractions must have been due to another, as yet unidentified, factor.

Amino acids were prepared in stock solutions adjusted to *p*H 8.0 with dilute NaOH. All amino acids used were the "natural" (−)-isomers. In earlier experiments, a mixture of amino acids was

*Present address: 64A, Portland Road, Edgbaston, Birmingham.

used; the final concentrations in 100 ml. of synthetic medium were: lysine, 4 mg.; alanine, 5 mg.; methionine, 3.5 mg.; threonine, 3.5 mg.; valine, 3.5 mg.; arginine, 3.25 mg.; leucine, 3.8 mg.; *iso*leucine, 2.6 mg.; phenylalanine, 1.3 mg.; tryptophane, 1.0 mg.; histidine, 0.6 mg. This mixture will be referred to as "the original amino acid solution." Subsequently, another amino acid mixture was used. On addition of this mixture to the synthetic medium, the final concentrations/100 ml. were: leucine, 11.7 mg.; lysine, 9.2 mg.; aspartic acid, 8.3 mg.; valine, 8.0 mg.; α-alanine, 7.5 mg.; histidine, 6.4 mg.; glutamic acid, 6.1 mg.; phenylalanine, 4.7 mg.; serine, 4.0 mg.; proline, 3.9 mg.; glycine, 3.5 mg.; arginine, 2.9 mg.; tyrosine, 2.7 mg.; threonine, 2.6 mg.; tryptophane, 1.3 mg.; methionine, 0.9 mg.; and *iso*leucine, 0.5 mg. This mixture will be referred to as the "globin amino acid solution" because the proportion of the individual amino acids in this mixture is the same as in globin. The amino acid analysis of human globin (performed by Dr. Lawrence Rosner, Laboratory of Vitamin Technology, Chicago, Illinois) yielded the following results (%): leucine, 13.0; lysine, 11.1; aspartic acid, 10.0; valine, 9.7; α-alanine, 9.1; histidine, 7.8; glutamic acid, 7.4; phenylalanine, 5.7; serine, 4.8; proline, 4.7; glycine, 4.3; arginine, 3.5; tyrosine, 3.3; threonine, 3.2; tryptophane, 1.6; methionine, 1.1; *iso*leucine, 0.6. When human globin was added to the synthetic medium, its final concentration was 75 mg./100 ml. This concentration was found to be optimal.

Proteolytic Activity.—Before the determination of proteolytic activity, adult schistosomes were dissected from the mesenteric and portal veins of mice infected 6 to 8 weeks previously with cercariae of *S. mansoni*. They were immediately placed in a chilled solution containing NaCl 1.3×10^{-1}M, KCl 2.8×10^{-3}M, $CaCl_2,2H_2O$ 3×10^{-4}M, $MgCl_2,6H_2O$ 5×10^{-3}M and buffered at *p*H 7.5 with the sodium salt of glycylglycine (final concentration 0.05M).

In the earlier experiments the schistosomes were transferred to a chilled all-glass homogenizer, together with 1.0 ml. of 0.9% saline for every 50 pairs of schistosomes, and ground up. The resulting suspension was stored in ice for a period not exceeding 2 hr. until required.

In later experiments, the enzyme was prepared from an acetone-dried powder of the schistosomes, which could be stored *in vacuo* at 0 to 3° for up to 2 weeks without loss of activity. In this manner, an enzyme of more constant potency could be prepared.

Protein concentrations of the enzyme preparations were determined by measuring the density of an appropriately diluted sample in 30% trichloracetic acid at 420 mμ in a Beckman spectrophotometer.

Preparation of Substrate.—The substrate used for the assay of proteolytic activity was bovine haemoglobin. 2 g. crystalline bovine haemoglobin was dissolved in 10 ml. of distilled water and dialysed. The concentration of haemoglobin in the dialysed solution was determined spectrophotometrically (Hawk, Oser, and Summerson, 1954), and was adjusted to 2.5% with a solution containing streptomycin, 150 mg., penicillin, 200 mg., and nystatin, 20 mg./l. After centrifugation at 3,000 rev./min. for 30 min. in a refrigerated centrifuge, the supernatant was removed and used as the substrate solution. It was stored in the frozen state.

Assay of Proteolytic Activity.—A modification of the method of Anson (1938) was used. 0.1 ml. of substrate solution was incubated with 0.1 ml. of 0.9% saline, 0.05 ml. homogenate, and 0.025 ml. 0.4 M-malate buffer (*p*H 3.8) at 37° for periods varying from 30 to 90 min. with mechanical shaking. Control experiments were always carried out in which the enzyme was incubated without substrate, and the substrate without enzyme. The reaction was stopped by the addition of 0.3 ml. of 5% trichloracetic acid, and the substrate was added to those tubes incubated with enzyme alone. The tubes were cooled in ice for 5 min. to allow precipitation of excess protein, and were then centrifuged at 3,000 rev./min. for 10 min. The supernatants were carefully transferred to chilled tubes, and aliquots were removed for amino acid determinations.

The amounts of amino acids liberated by the enzyme were determined in earlier experiments as their equivalent in tyrosine, using the reagent of Folin and Ciocalteu (1927), and in later experiments by the colorimetric ninhydrin method of Moore and Stein (1948) as modified by Cocking and Yemm (1954). With the ninhydrin method the results are expressed as μg. of free amino-nitrogen, calculated from a standard curve using alanine. Aliquots of the reaction mixtures were adjusted to *p*H 5.0 by the addition of a predetermined quantity of 0.5 N-NaOH before assay by the ninhydrin method.

Results

Synthetic Medium.—In an attempt to obtain a "balanced" amino acid mixture suitable for the survival of *S. mansoni in vitro*, addition of amino acids not present in the synthetic medium, as well as increases and decreases in the concentrations of individual amino acids, of groups of amino acids, or of the entire amino acid mixture, failed to prolong the survival of schistosomes. Since schistosomes degrade haemoglobin (Rogers, 1940; Halawani *et al.*, 1949), it is possible that they require only the amino acids from the digestion of globin. If this is so, the parasite should be adapted to utilize these amino acids in a medium containing the same proportions as in digested globin. Accordingly, an amino acid mixture with a qualitative and quantitative composition similar to that of globin was tested. Addition of this solution consistently resulted in a longer survival time than that in the original amino acid

solution (Table I) or of numerous modifications to the latter. Globin itself was less effective, but when globin was added to a medium containing the original amino acid solution, the worms survived for as long as in globin amino acid solution (Table I). These observations suggested that some of the nutritional requirements of schistosomes are met by the amino acids

TABLE I

EFFECT OF AMINO ACID MIXTURES AND OF GLOBIN ON THE MEAN SURVIVAL TIME OF *S. MANSONI* IN A SYNTHETIC MEDIUM

Means are calculated from 27 separate observations. Survival times in groups 2, 3, 4 and 5 were significantly longer than in Group 1 ($P<0.01$). There was no significant difference of survival times between Groups 4 and 2 or Groups 5 and 3.

Group	Medium	Mean±S.E. (Days)
1	No amino acids	2·1±0·17
2	Original amino acid solution	3·4±0·22
3	Globin amino acid solution	6·3±0·24
4	Globin	4·2±0·22
5	Globin+original amino acid solution	6·4±0·24

contained in globin and that these amino acids become available to the parasite within the host through the digestion of ingested red cells. This could be brought about by the action of one or several proteolytic enzymes present in the alimentary canal of the worms.

Proteolytic Enzyme.—Preliminary experiments showed that homogenates of *S. mansoni* were able to degrade haemoglobin at *p*H ranges between 2.0 and 5.0 with an apparent optimum activity in the region of *p*H 4.0. A sample of the homogenate boiled for 3 min. showed no proteolysis at *p*H 4.0, indicating the enzymatic nature of the process. Direct proportionality was observed between the rate of degradation of haemoglobin and the concentration of enzyme, and also between the degree of degradation and the time of incubation with a fixed concentration of enzyme.

A more accurate determination of the *p*H optimum was made using a more dilute preparation (15 worms in 0.6 ml.), to avoid high blank readings in the colorimetric determinations. Phosphate buffers were used between *p*H 2.9 and 3.2 and malate buffers for other *p*H values. The final concentrations of the buffers in the incubation mixtures were approximately 0.04 M. The *p*H values stated are those of the final incubation mixture, as determined with a *p*H meter. Fig. 1 shows that the enzyme has a sharply defined optimum *p*H of 3.9.

The effect of substrate concentration is shown in Fig. 2. There is optimal activity at a

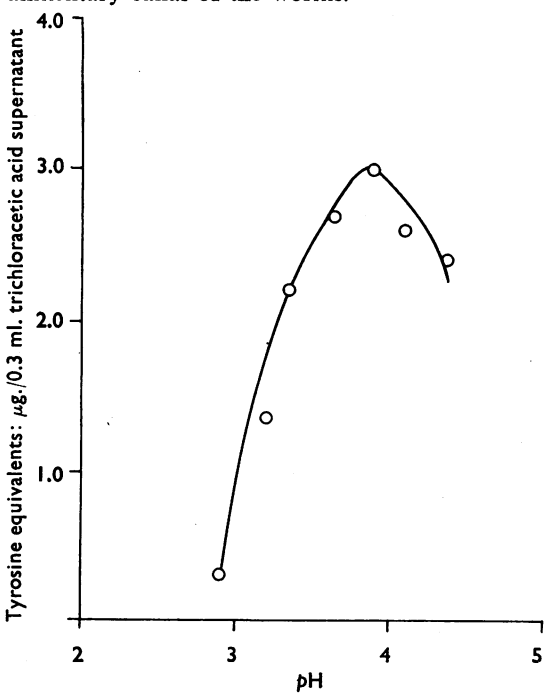

FIG. 1.—Effect of *p*H on activity of proteolytic enzyme of *S. mansoni*. 0·05 ml. of ground-up preparation was incubated for 45 min. at 37° with 1·0 mg. haemoglobin/ml.

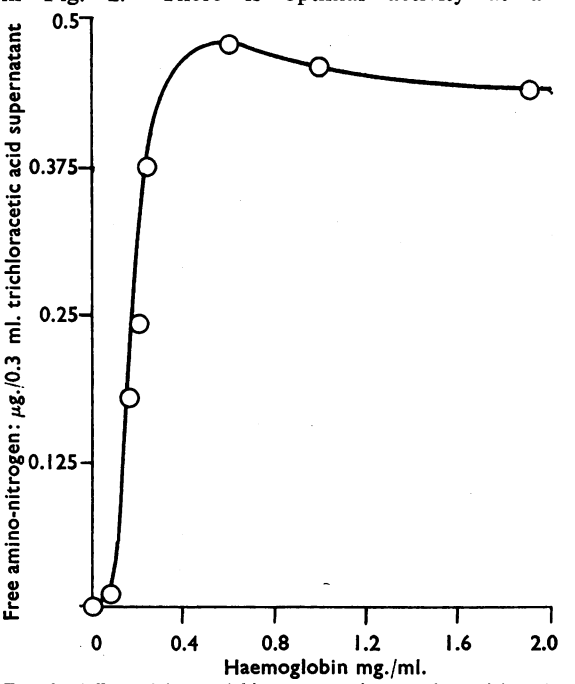

FIG. 2.—Effect of haemoglobin concentration on the activity of purified proteolytic enzyme from *S. mansoni*. 0·05 ml. of enzyme solution was incubated for 45 min. at 37° and *p*H 3·9 with 1 mg. haemoglobin/ml.

concentration of 0.5 mg. of haemoglobin/ml. incubation mixture. At 1.0 mg./ml., the activity is only slightly below the maximum; with concentrations of less than 0.5 mg./ml., the activity was reduced markedly.

Distribution of the Enzyme.—Both male and female schistosomes contained the enzyme. Separated male and female schistosomes were weighed, ground up in equal volumes of 0.45% saline, and assayed in duplicate at *p*H 3.9 with haemoglobin as the substrate. The female schistosomes contained 4.8 times the activity of the males/mg. of wet weight (Table II).

TABLE II
PROTEOLYTIC ACTIVITY OF MALE AND FEMALE SCHISTOSOMES

Activities expressed as μg. of free amino-nitrogen/0·3 ml. of trichloracetic acid supernatant released in 45 min. at 37° by 0·05 ml. of homogenates. Values are means of duplicate determinations.

	Wet Weight (mg.)	Activity	Activity/mg.	Ratio: Females/Males
Females ..	5.1	2·10	0·41	4·8
Males ..	9·4	0·80	0·085	

Purification of Enzyme.—An acetone-dried powder of freshly dissected schistosomes was found to possess 87.5% of the proteolytic activity found in the crude preparation, and to retain this for periods of up to 2 weeks *in vacuo* at 0 to 3°. The enzyme was found to be soluble and stable between *p*H 3.0 and 4.0. When a crude preparation of *S. mansoni* was adjusted to *p*H 3.8 and ultracentrifuged at 20,000 rev./min. (28,000 *g*) for 30 min., 45% of the initial activity was found in the clear supernatant. Under the same conditions, but in 0.02 M phosphate buffer at *p*H 3.0, 85% of the activity remained in the supernatant. When this procedure was applied to a suspension of an acetone-dried powder in 0.02 M phosphate buffer (*p*H 3.0), 82% of the original activity was recovered in the supernatant (Table III) though the protein concentration decreased to approximately one seventh of that in the suspension of acetone-dried powder. Therefore, the specific activity rose from 0.66 to 4.21.

TABLE III
PURIFICATION OF PROTEOLYTIC ENZYME FROM *S. MANSONI*

Activity expressed as μg. of free amino-nitrogen/0·3 ml. of trichloracetic acid supernatant. Incubated for 45 min. with 1·0 mg. of haemoglobin/ml. at *p*H 3·9 (37°). Specific activity was calculated as activity × 1,000/mg. protein/ml.

Step No.	Material	Activity	Protein (mg./ml.)	Specific Activity	Yield %
	Homogenate of schistosomes in 0·02 M phosphate (*p*H 3·0)	0·5	1·44	0·38	100·0
1	Acetone powder of schistosomes homogenized in 0·02 M phosphate (*p*H 3·0) ..	1·40	2·13	0·66	87·5
2	Supernatant after ultracentrifugation of 1. (20,000 rev./min. for 30 min.) ..	1·32	0·31	4·21	82·5
3	Supernatant after 2nd ultracentrifugation (39,000 rev./min. for 5 hr.)	1.06	0·13	7·80	66·5

Upon ultracentrifugation of the supernatant from Step 2 (see Table III) for 5 hr. at 39,000 rev./min. (108,000 *g*), 66.5% of the enzyme remained in the supernatant, although the protein concentration was less than half that in Step 2. The specific activity rose to 7.8, representing a twenty-fold purification compared with the crude preparation. The material from Step 3 will be referred to as "the purified enzyme." After partial purification the rate of amino-nitrogen production remained proportional to the enzyme concentration (Fig. 3) and the time of incubation (Fig. 4).

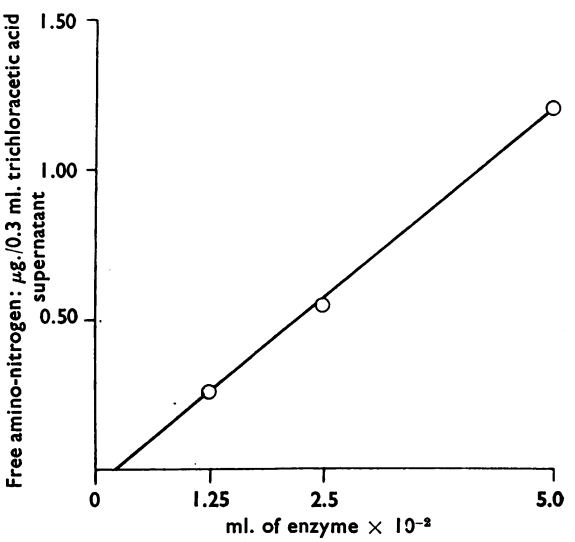

FIG. 3.—Relationship between concentration of proteolytic enzyme of *S. mansoni* and rate of haemoglobin degradation. Incubation was for 45 min. at 37° at *p*H 3·9 with 1·0 mg. of haemoglobin/ml.

Substrate Specificity of the Enzyme.—Table IV summarizes the results of substrate specificity studies using the purified enzyme. The enzyme showed a marked specificity for haemoglobin and globin, with both of which maximal proteolysis occurred at *p*H 3.9. No significant proteolysis was observed at any *p*H with any serum protein tested. A possible exception to this

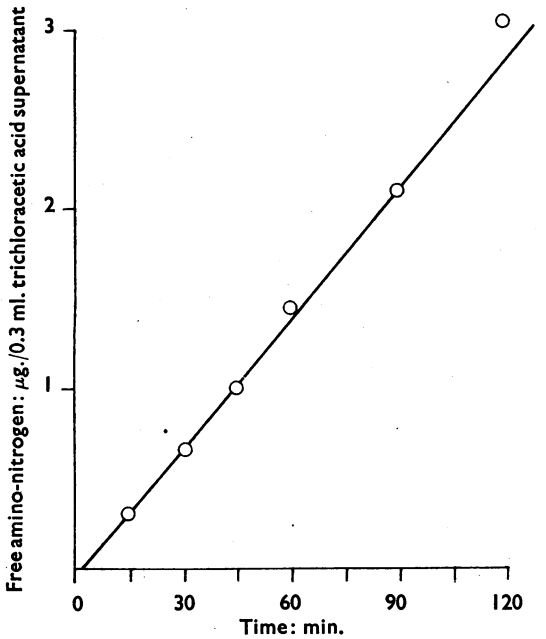

FIG. 4.—Relationship between time of incubation and rate of haemoglobin degradation. 0·05 ml. of homogenate was incubated at 37° and *p*H 3·9 with 1·0 mg. of haemoglobin/ml.

is bovine serum mercaptalbumen at *p*H 7.7. The last column of Table IV shows the amount of free amino-nitrogen released from haemoglobin by the enzyme under optimal conditions. These samples served as controls to ensure that the different batches of enzyme were active.

TABLE IV

SUBSTRATE SPECIFICITY OF PROTEOLYTIC ENZYME FROM *S. MANSONI*

The values are given as μg. of free amino-nitrogen/0·3 ml. of trichloracetic acid supernatant after 45 min. incubation at 37° with 0·05 ml. of purified enzyme.

*p*H	Substrate (1·0 mg./ml.)							
	Bovine Haemoglobin	Bovine Globin	Bovine Serum γ-Globulin	Porcine Serum γ-Globulin	Bovine Serum Albumen	Bovine Serum Mercaptalbumen	Bovine Serum Glycoprotein Fract. VI	Control Haemoglobin *p*H 3·9
2·50	0·0	0·10	0·0	0·10	0·0	0·0	0·0	1·50
3·90	1·70	1·35	0·13	0·15	0·0	0·13	0·0	1·70
5·50	0·39	0·50	0·0	0·01	0·0	0·0	0·0	1·20
6·60	0·0	0·0	0·0	0·01	0·20	0·0	0·25	1·50
7·70	0·60	0·10	0·0	0·0	0·0	0·50	0·0	2·00

Experiments were also made with two basic proteins, histone and protamine, and with lactoglobulin. These proteins could not be precipitated with trichloracetic acid in the normal manner, and gave very high blank readings if left in solution. The addition of 0.25 ml. of a 5% aqueous solution of zinc sulphate was found to precipitate these proteins; the amino acids remained in solution. None of these substrates was degraded by the enzyme at *p*H 3.9.

The reported survival of *S. mansoni* in undiluted horse serum (Robinson, 1956) raised the possibility that some serum protein, not tested above, may be degraded by the schistosomes. Accordingly, both the purified enzyme and the crude homogenate were tested for proteolytic activity using haemoglobin and dialysed human serum as substrates at *p*H 3.9, 6.0, and 8.0. Table V shows that free amino-nitrogen was produced

TABLE V

EFFECT OF PURIFIED ENZYME AND OF CRUDE HOMOGENATE OF *S. MANSONI* UPON DIALYSED SERUM

Activities are expressed as μg. of free amino-nitrogen 0·3 ml. of trichloracetic acid supernatant in 45 min. at 37°.

Substrate	Activity					
	Purified Enzyme			Crude Preparation		
	*p*H			*p*H		
	3·9	6·0	8·0	3·9	6·0	8·0
Haemoglobin	2·25	0·10	0·20	2·40	0·60	0·10
Human serum (dialysed)	0·00	0·30	0·35	0·30	0·00	0·00

from haemoglobin only at *p*H 3.9 (purified and crude enzymes), and at *p*H 6.0 (crude enzyme only). With neither enzyme was there a significant production of free amino-nitrogen from dialysed serum. The previously undetected degradation of haemoglobin at *p*H 6.0 by the crude preparation suggests the existence of a second proteolytic enzyme.

DISCUSSION

A solution containing amino acids in the same proportions as in globin increased the survival of *S. mansoni* in a synthetic medium to a greater extent than did other amino acid mixtures, and it therefore appears that the parasite is well adapted to utilize amino acids obtained from the digestion of haemoglobin. This process may represent a major source of essential amino acids for the worms; after the ingestion of red cells, the schistosomes deposit haematin crystals in their alimentary canals (Rogers, 1940), indicating that the intact worms digest haemoglobin. Furthermore, the activity of the proteolytic enzyme which degrades haemoglobin is approximately five times as high in the female as in the male worms (Table II). While the alimentary tract of both female and male schistosomes is of the same size, the males are three to five times as heavy as the females, due to the much greater weight of the musculature of

the males. Therefore, the higher proteolytic activity/mg. exhibited by preparations from female worms is consistent with the view that this enzyme is located in the alimentary canal. It remains to be determined whether the *p*H within the intestinal canal of the schistosome is at or near the optimum for the enzyme.

Undegraded globin was less effective in prolonging the survival of schistosomes *in vitro* than were the free amino acids contained in globin. Possibly, in the synthetic medium used, the activities of the proteolytic enzymes of the worms were lower than in the natural habitat of adult schistosomes. Addition of globin to the original synthetic medium prolonged survival; thus, even *in vitro,* some digestion of haemoglobin must have occurred. It is conceivable that in their natural habitat, schistosomes are able to utilize the free amino acids present in blood plasma as well as those produced by the digestion of haemoglobin.

The high degree of specificity of the proteolytic schistosome enzyme for haemoglobin and the inability of crude and purified preparations of the worms to hydrolyse any serum proteins to a significant degree (Table V) suggest that haemoglobin is the only host-protein which can supply schistosomes with amino acids. Therefore, interference with haemoglobin degradation by the worms might deprive the parasite of an essential source of amino acids and thereby provide an opportunity for the development of schistosomicidal agents. Furthermore, if, by the use of simple peptides of known structure, it can be established which bonds are hydrolysed by this proteolytic enzyme, the latter could serve as a tool for the elucidation of the amino acid sequence in the globin molecule.

After this paper had been submitted for publication, Cheever and Weller (1958) reported that addition of red cells, haemoglobin or globin to tissue culture media increased the survival of *S. mansoni in vitro,* while haemin had no effect. These findings provide additional support for our conclusions regarding the nutritional significance of the amino acids of globin for *S. mansoni.*

It is a pleasure to express appreciation to Mr. J. Hawkins and to Mr. H. Ruppender for able technical assistance. The authors are indebted to Dr. R. B. Pennell of Messrs. Sharp and Dohme Ltd. for supplies of human globin.

The work was carried out during the tenure of a Riker Research Fellowship (1956–7) by A. R. T. It was supported in part by the Research and Development Division of the Office of the Surgeon-General, Department of the Army, under contract No. DA-49-007-MD-413 and, in part, by a grant (No. E-668) from the National Institutes of Health, United States Public Health Service.

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