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Proline Synthesis by *Schistosoma mansoni* Egg Granulomas

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Abstract

To clarify the mechanism responsible for the increased proline pool in the fibrotic liver of ICR female mice infected with *Schistosoma mansoni*, a capacity of the egg granulomas for proline production was examined. The native egg granulomas could produce and release proline during *in vitro* incubation in amino acid-free medium. Such a proline formation was markedly stimulated by adding amino acid mixture, arginine, ornithine or Δ^1-pyrroline-5-carboxylate (P5C). Product analysis on the egg granulomas incubated with ^{14}C-arginine demonstrated conversion of arginine to proline or to glutamic acid through two intermediates, ornithine and P5C. In contrast, a conversion of glutamic acid to proline was not detected under the present experimental conditions. Moreover, the tracer experiment on the granulomas using ^{14}C-proline demonstrated a conversion of proline to glutamic acid through P5C. All enzymatic activities involved in arginine-derived proline formation and in proline degradation, i.e., arginase [EC 3.5.3.1], ornithine-δ-transaminase [EC 2.6.1.13], pyrroline-5-carboxylate reductase [EC 1.5.1.2], glutamic-γ-semialdehyde dehydrogenase [EC1.5.1.12] and proline oxidase [EC 1.4.3.2], were detected in the crude extract of egg granulomas. These findings suggest that active conversion of arginine to proline by the egg granulomas may be responsible for the increased free proline pool in fibrotic liver of mice with schistosomiasis mansoni.

Key words: *Schistosoma mansoni*, Egg granuloma, Liver fibrosis, Proline synthesis, Proline degradation, Free proline

Introduction

Hepatic schistosomiasis is probably the most prevalent chronic liver disease in the world (Dunn and Kamel, 1981). *Schistosoma mansoni* eggs trapped in liver lead to a granulomatous response and ultimately to hepatic fibrosis. In human and murine schistosomiasis mansoni, an extraordinary accumulation of hepatic collagen (Dunn *et al.*, 1979; Tanabe *et al.*, 1991) occur as the result of various molecular and biochemical changes such as an increased prolyl hydroxylase activity (Dunn *et al.*, 1978a), an increased collagen synthesis (Dunn *et al.*, 1977; Dunn *et al.*, 1979), an elevation of type I and IV procollagen steady-state mRNA levels, and an increased transcriptional rates of these procollagen genes (Weiner *et al.*, 1987; Zern *et al.*, 1983).

Proline and hydroxyproline are major amino acid components of collagen. The supply of free proline within collagen-synthesizing compartment is thought to be an important factor in regulating the rate of collagen synthesis (Dunn *et al.*, 1977; Henley *et al.*, 1977; Kershenobich *et al.*, 1970; Rojkind and Diaz de Leon, 1970). Increased pool of hepatic free proline has been observed in virtually all sorts of liver fibrosis (Dunn *et al.*, 1977; Dunn *et al.*, 1979; Kershenobich *et al.*, 1970; Rojkind *et al.*, 1977; Tanabe *et al.*, 1991). Therefore, clarification of the mechanisms responsible for elevation of hepatic free proline level would have the possibility of preventive or therapeutic applications for liver fibrosis.

Proline may be synthesized either from arginine (Dunn *et al.*, 1978; Zinker and Rojkind, 1972) or glutamate (Rojkind and Diaz de Leon, 1970; Sallach *et al.*, 1951; Shen and Strecker, 1975) depending on tissue and its metabolic state. Although Dunn *et al.* (1978b) have already demonstrated an increased proline synthesis in the liver slices prepared from *S. mansoni*-infected mice, biochemical and cellular

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mechanisms responsible for this process remains unknown. In our previous study (Tanabe *et al.*, 1991) a transient elevation of hepatic free proline level in the infected mice has been observed during 7th to 11th week of infection, when host granulomatous reaction is most prominent. Because this finding led us to conceive a close correlation between elevation of hepatic free proline level and host granulomatous reaction, we attempted to clarify the capacity of egg granulomas for *de novo* proline synthesis and proline degradation. Moreover, quantitative analysis were also conducted to clarify the mechanism responsible for increased free proline pool in the livers of *S. mansoni*-infected mice.

Materials and Methods

Experimental animal and parasite

ICR female mice (8 to 9 weeks of age), which are high responder concerning granulomatous and fibrotic reactions to schistosome eggs (Cheever *et al.*, 1983), were used for this study. Mice were fed on ordinary mouse pellet (Oriental Yeast Co., Tokyo, Japan) *ad libitum.* A Puerto Rican strain of *S. mansoni*, which had been maintained in our laboratory by serial passage in vector snails (*Biomphalaria glabrata*), was used in this study.

Chemicals

L-[U-^{14}C]Arginine, 147 mCi per mmole, was obtained from ICN Radiochemicals Inc. (Irvine, CA, USA). L-[U-^{14}C]Glutamic acid, 266 mCi per mmole, and L-[U-^{14}C]proline, 293 mCi per mmole were obtained from New England Nuclear (Boston, Mass, USA). L-[U-^{14}C]Glutamic acid was further purified by ion-exchange chromatography (Niwaguchi *et al.*, 1965). Radiochemical purity determined by ion-exchange chromatography was 98% for arginine, 96% for proline, and 95% for glutamic acid after the purification. Urease from jack beans, ornithine decarboxylase from *Escherichia coli*, and 1-fluoro-2,4-dinitrobenzene were purchased from Sigma Chemicals Co. (St Louis, MO, USA). AG-2 (acetate form, 8% cross-linked, 200 to 400 mesh) and AG-50W (H^+ form, 8% cross-linked, 200 to 400 mesh) ion-exchange resins were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Hank's balanced salt solution (Hank's BSS) without pH indicator was obtained from Nissui Chemicals (Tokyo, Japan). A fifty-times concentrated amino acid mixture for minimal essential medium (Eagle) was obtained from Gibco Oriental Co. (Tokyo, Japan). Preparation of Δ^1-pyrroline-5-carboxylate (P5C) hydrazone from D,L-hydroxylysin and regeneration of P5C from P5C hydrazone were conducted according to the previous method (Mezl and Knox, 1976). Optical rotation of synthesized P5C was zero by measurement with a Jasco DIP-360 polarimeter (Japan Spectroscopic Co., Tokyo, Japan). Other chemicals were of the highest purity commercially available.

Isolation of S. mansoni eggs and the egg granulomas

The eggs of *S. mansoni* were isolated from the livers of mice 9 weeks after infection with 250 cercariae according to the previous methods (Coker and Lichtenberg, 1956; Isseroff *et al.*, 1983). The isolated eggs were washed 7 times with Hank's BSS containing 1.5% NaCl, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The viability of eggs were evaluated microscopically on the basis of movement of flame cells. Batches of eggs used for experiments had a viability of 61 to 73%.

The granulomas were isolated from the livers of infected mice 9 weeks after infection with 50 cercariae. Because hepatocytes are known to metabolize proline, the method for granuloma isolation was preliminarily examined to minimize a contamination with hepatocytes. Consequently, the following method seemed to be most appropriate on the basis of histological examination; three mouse livers (10 to 12 g wet weight) were weighed and cut into small pieces. These were placed in 60 ml of sterilized Hank's BSS supplemented with 25 mM HEPES (pH 7.2), penicillin (100 IU/ml) and streptomycin (100 μg/ml), referred to as Hank's BSS-HA. The mixture was homogenized with a Warring blender for 20 sec at full power. The homogenate was strained through a stainless filter (10 mesh) to remove large debris. The granulomas were rinsed 6 times by decantation with 60 ml of sterilized Hank's BSS-HA for 30 sec. Egg granulomas employed for *in vitro* incubation studies were isolated aseptically (Tanabe *et al.*, 1991). For preparation of acid-denatured granulomas, the isolated granulomas were

suspended in ice-chilled 10% TCA (w/v) for 20 min. After washing with the Hank's BSS-HA several times, they were employed for the incubation experiment.

All isolations and assays were carried out at 4°C except for determination of pyrroline-5-carboxylate reductase activity, which was conducted at 25°C due to its cold lability (Herzfeld *et al.*, 1977).

Procedure of in vitro incubation of the eggs and egg granulomas

The isolated eggs and granulomas were processed further according to the purpose of experiments as follows.

(1) One hundred and thirty granulomas were incubated at 37°C in 3 ml of the Hank's BSS-HA medium in the presence or absence of amino acid under an atmosphere of 5% CO_2 and 95% air to evaluate their capacity for proline production. Five or ten thousand viable eggs were also incubated under the same incubating condition as above except that NaCl concentration in the medium was increased to 1.5% to avoid hatching of the eggs. At various time intervals, the incubation was stopped by adding ice-chilled 50% TCA (w/v) to a final concentration of 5%. The suspension was homogenized with a glass homogenizer, and the homogenate was kept on ice for 20 min. TCA-soluble supernatants obtained by centrifugation at 800g for 15 min at 4°C were used for amino acid analysis. TCA-insoluble material was used for protein and DNA determinations.

(2) For tracer experiments, 400 egg granulomas were incubated in 9 ml of the Hank's BSS-HA medium containing 20 μCi of ^{14}C-arginine or ^{14}C-glutamic acid. In both experiments, unlabelled amino acid was added to provide 0.1 mM concentration. In other incubations both of unlabelled arginine and glutamic acid were added to provide 0.1 mM concentration in the incubation medium containing either of ^{14}C-labelled amino acids. To measure proline degradation, the same number of egg granulomas were incubated as above with 20 μCi of ^{14}C-proline in the presence of 0.1 mM unlabelled proline.

The aliquot of these incubation media, usually 200 μl, was taken at 0, 1, 2, 4 and 6 hr post-incubation, mixed with an equal volume of 10% TCA (w/v) containing one micromole each of unlabelled arginine, ornithine, glutamic acid and proline, and kept on ice for 20 min. The TCA-soluble supernatant fluid was isolated by centrifugation and used for determination of amino acids. After 6 hr of incubation, ice-cold 50% TCA (w/v) was added to the remaining incubation medium to produce a final concentration of 5%. Each sample was further processed as above to isolate the TCA-soluble and insoluble fractions. These were used for determinations of P5C radioactivity, and for protein and DNA, respectively.

Amino acid analyses

(1) Separation of amino acids: Proline and ornithine were separated by ion-exchange chromatography with AG-50W column (0.5×2.5 cm). Separation of radioactive arginine, ornithine, proline and glutamic acid was done by either AG-50W (1×15 cm) or AG-2 (1×10 cm) columns (Shen and Strecker, 1975). The fractions containing proline, ornithine, arginine and glutamic acid were pooled separately, and evaporated to dryness *in vacuo* at 45°C.

(2) Colorimetric and enzymatic determination of amino acids: Proline was determined colorimetrically without Permutit treatment (Troll and Lindsley, 1955). Ornithine concentration was estimated by the enzymatic procedure using ornithine decarboxylase and 1-fluoro-2,4-dinitrobenzene (Hutzler, 1974).

(3) The radioactivity of proline was determined after further purification using thin-layer chromatography with cellulose (Merck, Tokyo, Japan) (Shen and Strecker, 1975). Ornithine radioactivity was determined by counting radioactive CO_2 liberated (Ichiyama *et al.*, 1970) during ornithine decarboxylating reaction (Hutzler, 1974). Measurement of radioactive arginine and glutamic acid was done by the method of Shen and Strecker (1975). Radioactivity of urea was measured by counting radioactive CO_2 liberated during the urease reaction (Schimke, 1970) as above. Isolation and measurement of radioactive P5C were performed according to Phang *et al.* (1975). For counting the radioactivities of these amino acids, an aliquot (200 μl) of the aqueous solution containing each amino acid was mixed with 10 ml Aquasol-2 (New England Nuclear). To measure radioactivity of ornithine and

urea, the strips of filter paper which absorbed radioactive CO_2 were immersed in 10 ml of the same scintillation fluid as above. The radioactivity was measured with a scintillation spectrometer (Model LSC-700, Aloka, Tokyo, Japan). Counting efficiency, estimated by the use of internal standard, was 83.3%.

The recovery rates of radioactive proline, ornithine, arginine and glutamic acid standards by our methods were 85.4, 82.7, 87.0 and 81.9%, respectively. No adjustment was made for loss of radioactivity based on this recovery rate. The radioactivity of proline, glutamic acid and P5C were multiplied by 1.2 to correct for loss of one-sixth of the carbon radioactivity of arginine. For ornithine and urea, radioactivities were also multiplied by 6.

Enzyme assay

Liver specimens and egg granulomas for enzyme assays were prepared from uninfected controls and *S. mansoni*-infected mice 9 weeks after infection with 50 cercariae.

Arginase, ornithine-δ-transaminase and proline oxidase: Preparation of the homogenates of liver specimens and egg granulomas, and determination of enzymatic activities were performed according to the previous method (Tanabe *et al.*, 1989; Tanabe *et al.*, 1991).

Pyrroline-5-carboxylate reductase: Approximately 100 mg wet weight of liver specimen and 0.4 ml packed volume of the egg granulomas were homogenized at 25°C for 1 min in 2 ml of the disrupting solution (Herzfeld *et al.*, 1977). The supernatant obtained by centrifugation at 5,500 g for 15 min at 25°C was employed for the measurement of enzymatic activity at 37°C by colorimetry (Herzfeld *et al.*, 1977).

Glutamic-γ-semialdehyde dehydrogenase: Approximately 200 mg wet weight of liver specimen and 0.4 ml packed volume of the egg granulomas were homogenized at 4°C for 1 min in 2 ml of the disrupting solution (Herzfeld *et al.*, 1977). The supernatant solution isolated by centrifugation at 5,500 g for 20 min at 4°C was employed for the measurement of enzymatic activity at 20°C by spectrophotometry (Herzfeld *et al.*, 1977).

Protein and DNA determinations of TCA-insoluble precipitates

Aliquots of the homogenates prepared for the enzyme assays were mixed with an equal amount of 10% TCA (w/v), and then TCA-insoluble precipitates were separated by centrifugation. These TCA-insoluble precipitates and those obtained from the incubation and tracer experiments were washed twice more with ice-cold 10% TCA (w/v) and extracted with organic solvents according to Schneider's method (1945). The residual material was dried *in vacuo* at room temperature, and was homogenized in 0.01N HCl solution to make fine suspension. The suspension was completely dissolved by mixing with the same amount of 2N NaOH, and then protein was determined by biuret reaction (Gornall *et al.*, 1949) with bovine serum albumin (Fraction V) as a standard. DNA was extracted according to the method of Schneider (1945) and determined by the diphenylamine reaction (Burton, 1956) using calf thymus DNA as a standard.

Statistics

Statistical analysis of the data was done with Student's *t*-test (pairs). Differences with probability (P) less than 0.05 were considered significant.

Results

Proline and ornithine production by the eggs and egg granulomas

To clarify the capacity of the egg granulomas for *de novo* proline synthesis, isolated egg granulomas were incubated in the Hank's BSS-HA medium. As shown in Fig. 1 (line a), proline and ornithine concentration increased progressively with time. However, acid-denatured egg granulomas lost this capacity (Fig. 1, line b). Addition of the amino acid mixture markedly stimulated the production of ornithine and proline by the egg granulomas (Fig. 1, line c). However, addition of dialyzed fetal calf serum (1%, v/v) or bovine serum albumin (1 mg/ml) did not show any appreciable effect in the presence of amino acid mixture. After 6 hr of incubation, the concentration of proline and ornithine in the samples of Hank's BSS-HA medium were almost identical with those in the supernatant fraction of the homogenate of incubation mixture, which indicates

a equilibration of these two amino acids between medium and egg granulomas.

When 5,000 (Fig. 1, line e) or 10,000 (Fig. 1, line d) viable eggs were incubated in the Hank's BSS-HA medium containing 1.5% NaCl and the amino acid mixture, concentration of proline and ornithine in the TCA-soluble supernatant fluid increased in proportion to the number of eggs incubated. A net increase in the concentration of proline and ornithine by 10,000 viable eggs during 6 hr incubation were 7.5 and 54 nmoles per vessel, which were 15% and 1.5% of those in the incubation of 130 egg granulomas, respectively. Moreover, because 930±98 eggs (N=10) were recovered from 130 egg granulomas by chemical digestion with 4% KOH (w/v), a net production of proline and ornithine by schistosome eggs seemed negligible as compared to

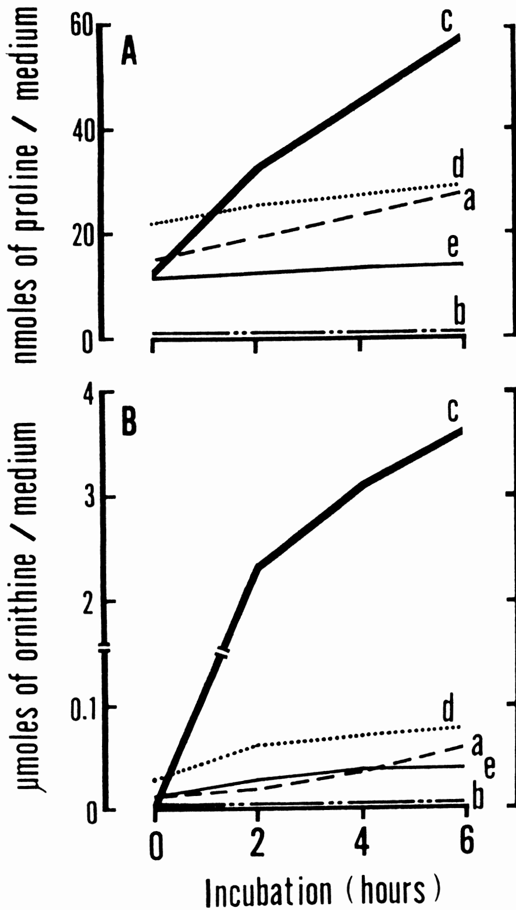

Fig. 1 *In vitro* production of proline and ornithine by *Schistosoma mansoni* eggs and the egg granulomas.
One hundred and thirty egg granulomas were incubated for 6 hr in 3 ml of Hank's BSS-HA medium in the absence (a) or presence of amino acid mixture (c). The same number of TCA-denatured egg granulomas were incubated in the Hank's BSS-HA medium containing amino acid mixture (b). Five (d) and ten (e) thousands of *S. mansoni* viable eggs were incubated in the Hank's BSS-HA medium containing 1.5% sodium chloride and amino acid mixture. Preparation and determination of proline and ornithine were performed according to the method described in the text. Each datum of proline (A) and ornithine (B) represents the mean concentration of three incubation vessels.

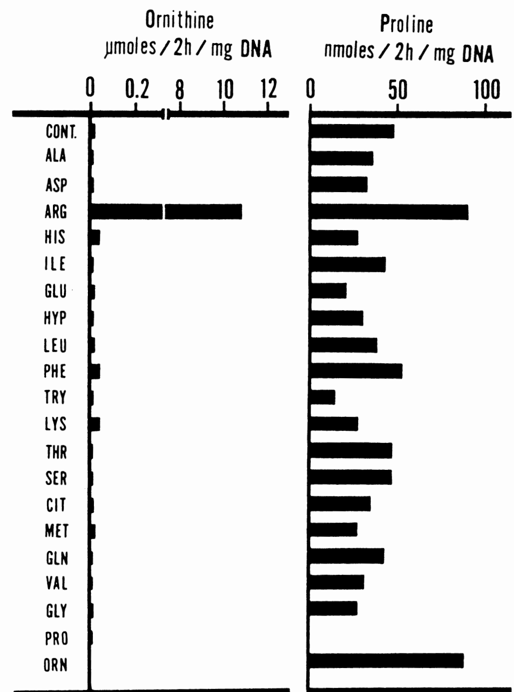

Fig. 2 Effect of amino acids on proline and ornithine production by *Schistisoma mansoni* egg granulomas.
One hundred and thirty egg granulomas were incubated for 2 hour in 3 ml of the Hank's BSS-HA medium containing each amino acid at a concentration of 3 mM. Each datum represents the mean concentration of three incubation vessels.
Abbreviations were used as follows: Alanine (ALA); Arginine (ARG); Aspartic acid (ASP); Glutamine (GLN); Glutamic acid (GLU); Glycine (GLY); Histidine (HIS); Isoleucine (ILE); Leucine (LEU); Lysine (LYS); Methionine (MET); Phenylalanine (PHE); Proline (PRO); Serine (SER); Threonine (THR); Tryptophan (TRY); Valine (VAL); Ornithine (ORN); Citrulline (CIT); Hydroxyproline (HYP).

the capacity of egg granulomas.

Effect of amino acids on proline production by the egg granulomas

Each of twenty amino acids was examined for their effect on production of proline and ornithine by the granulomas. Among them, arginine stimulated proline and ornithine production, and ornithine stimulated proline production (Fig. 2). Moreover, when the egg granulomas were incubated for 2 hr in the presence of 3 mM arginine, the concentration of proline and ornithine in the incubation medium increased in proportion to the number of egg granulomas in the range of 25 to 200. However, neither glutamic acid nor glutamine stimulated proline and ornithine synthesis by the egg granulomas.

Effect of various concentration of arginine, ornithine and P5C on proline production by the egg granulomas

The effect of various concentration of arginine, ornithine and P5C was examined under the same incubating condition as above. Proline production by the egg granulomas at various concentrations of arginine or ornithine clearly showed a saturation profile with substrate (Fig. 3). The rate of proline production in the presence of 5mM arginine or ornithine was approximately 100 nmoles/mg DNA/2 hour. In contrast, proline production by the egg granulomas increased in proportion to P5C concentration. The synthetic rate of proline by the granulomas in the presence of 1mM P5C was almost 3-times higher than those in the presence of 5mM arginine or ornithine.

Product analysis during in vitro incubation of the egg granulomas with radiolabelled amino acids

Product analysis was conducted during *in vitro* incubation of the egg granulomas with ^{14}C-arginine or ^{14}C-glutamic acid. When the egg granulomas were incubated in the medium containing ^{14}C-arginine, free arginine decreased rapidly (Fig. 4). Only 10% of its radioactivity remained in the medium when the incubation was stopped. On the other hand, ornithine concentration in the medium in-

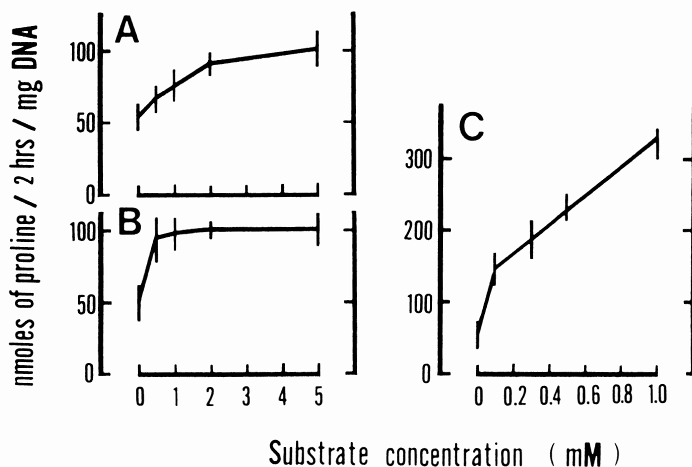

Fig. 3 Effect of various concentration of arginine, ornithine or Δ^1-pyrroline-5-carboxylate in the medium on the proline production by *Schistosoma mansoni* egg granulomas.
One hundred and thirty egg granulomas were incubated for 2 hr in the Hank's BSS-HA medium containing various concentrations of arginine (A), ornithine (B) or P5C (C). Each point represents the mean cocnentration of proline in three incubation vessels. Vertical line represents the standard deviation.

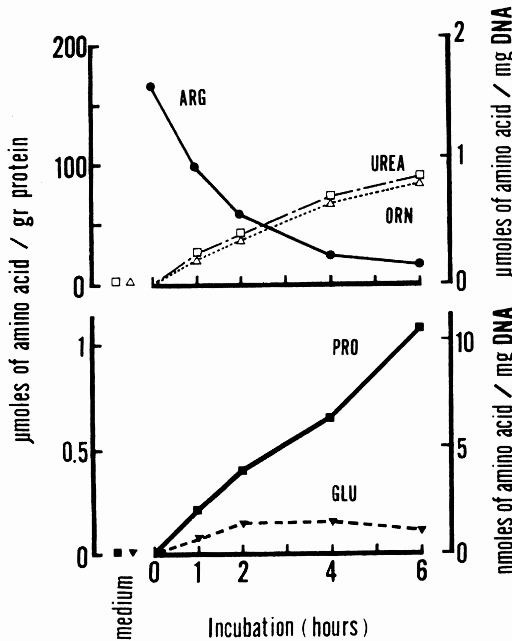

Fig. 4 Changes in the concentration of free arginine, ornithine, urea, glutamic acid and proline during *in vitro* incubation of *Schistosoma mansoni* egg granulomas with ^{14}C-arginine.
Four hundred egg granulomas were incubated for 6 hr in 9 ml of the Hank's BSS-HA medium containing ^{14}C-arginine. Preparation and determination of radioactive amino acids were performed according to the method described in the text. Each point represents the mean concentration of each amino acid in three incubation vessels.
Abbreviations used in this figure are given in the legend to Fig. 2.

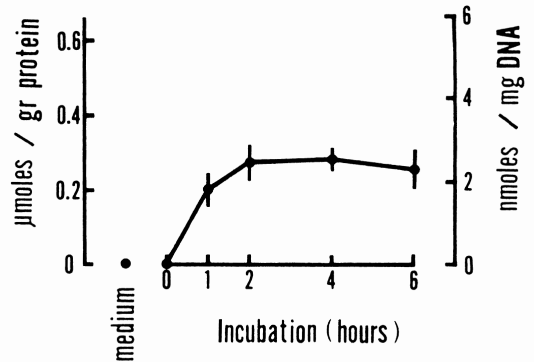

Fig. 5 Change in the concentration of free glutamic acid during *in vitro* incubation of *Schistosoma mansoni* egg granulomas with ^{14}C-proline.
Four hundred egg granulomas were incubated for 6 hr in 9 ml of the Hank's BSS-HA medium containing ^{14}C-proline. Each point represents the mean concentration of three incubation vessels. Vertical line represents the standard deviation.

creased progressively in parallel with that of urea. Approximately 58% of utilized arginine was converted to ornithine and urea. The concentration of arginine-derived proline also progressively increased. Approximately 1.17±0.21 μmoles of proline per g of granuloma protein was produced during 6 hr incubation. An additional arginine-derived radioactive material was identified as glutamic acid. The synthetic rate of glutamic acid from arginine was significantly lower than that of arginine-derived proline. At the end of incubation, 0.11±0.03 μmoles of glutamic acid per g of granuloma protein was present in the medium. Moreover, radioactive P5C (0.23±0.04 μmoles per g of granuloma protein) was demonstrated. The similar result was also obtained by the incubation of egg granulomas with ^{14}C-arginine in the medium containing 0.1 mM unlabelled arginine and glutamic acid.

In contrast, neither radioactive proline nor P5C was detected during *in vitro* incubation of the egg granulomas with ^{14}C-glutamic acid. The same result was noted with egg granulomas incubated with ^{14}C-glutamic acid in the medium containing 0.1 mM unlabelled glutamic acid and arginine.

Proline degradation by the egg granulomas

To clarify the capacity of egg granulomas for proline degradation, 400 egg granulomas were incubated with ^{14}C-proline in the medium containing 0.1 mM unlabelled proline (Fig. 5). Almost 92% of total radioactivity added as ^{14}C-proline remained in the medium after 6 hour incubation. Concentration of P5C and glutamic acid at the end of incubation were 0.067 and 0.251 μmoles per g of granuloma protein, respectively.

Activities of proline-forming and -degrading enzymes in the egg granulomas

The activities of proline-forming and -degrading enzymes in the egg granulomas were assayed and compared with those in the liver homogenates pre-

Table 1 The activities of proline-forming and -degrading enzymes in the livers of uninfected controls and *Schistosoma mansoni*-infected mice, and in the egg granulomas

Enzyme	Normal liver (a)	Fibrotic liver (b)	Egg granuloma (c)	Significance of enzyme activity difference		
				(a) vs. (b)	(a) vs. (c)	(b) vs. (c)
Arginase	(7)	(7)	(5)			
nmoles/min/mg pro.	8160±740	6950±580	5120±410	P<0.01	P<0.001	P<0.001
μmoles/min/mg DNA	432±39	209±17	56±5	P<0.001	P<0.001	P<0.001
Ornithine-δ-transaminase	(4)	(5)	(5)			
nmoles/min/mg pro.	7.6±1.5	7.9±2.0	2.8±1.0	N.S.	P<0.001	P<0.001
nmoles/min/mg DNA	398±78	246±62	33±9	P<0.02	P<0.001	P<0.001
Pyrroline-5-carboxylate reductase	(5)	(5)	(5)			
nmoles/min/mg pro.	218±8	124±12	50±7	P<0.001	P<0.001	P<0.001
nmoles/min/mg DNA	8018±382	2544±562	609±67	P<0.001	P<0.001	P<0.001
Glutamic-γ-semialdehyde dehydrogenase	(5)	(5)	(5)			
nmoles/min/mg pro.	50±4	22±2	7±3	P<0.001	P<0.001	P<0.001
nmoles/min/mg DNA	2712±106	645±97	78±31	P<0.001	P<0.001	P<0.001
Proline oxidase	(4)	(5)	(5)			
nmoles/min/mg pro.	16.7±0.5	8.9±1.7	2.3±0.9	P<0.001	P<0.001	P<0.001
nmoles/min/mg DNA	852±27	285±54	27±10	P<0.001	P<0.001	P<0.001

Enzymatic activity was expressed as nmoles or μmoles of reaction product per one min per mg protein or DNA. Each datum represents the mean±standard deviation. Number of animals examined were expressed in parenthesis.

pared from uninfected controls and *S. mansoni*-infected mice (Table 1). The activities of three enzymes involved in arginine-derived proline formation, i.e., arginase, ornithine-δ-transaminase and pyrroline-5-carboxylate reductase, and of two proline-degrading enzymes, i.e., proline oxidase and glutamic-γ-semialdehyde dehydrogenase, were demonstrable in the crude extract of egg granulomas. The specific activities of three proline-forming enzymes in the granulomas were significantly lower than those in normal and fibrotic livers.

The specific activities of two proline-degrading enzymes in the granulomas were 14% of those in normal liver. In addition, the ratio of the specific activity of proline oxidase to that of ornithine-δ-transaminase in the granulomas was significantly lower than that of normal liver.

Discussion

The present investigation demonstrated that the egg granulomas actively produce and release proline during *in vitro* incubation with arginine, ornithine or P5C. Although active production of proline by schistosome eggs (Isseroff *et al.*, 1983) and adults (Senft, 1965) have been reported, the present study indicates that host cells, rather than schistosome eggs, might play an essential role in proline formation by the egg granulomas. Tracer experiments with radiolabelled amino acids showed a conversion of arginine to proline, a rapid formation of urea and ornithine, and an appearance of P5C in the egg granulomas. All enzymatic activities involved in arginine-derived proline formation were detected in the crude extract of egg granulomas. Moreover, because there was little contamination with hepatocytes in the isolated egg granulomas prepared by our method, the egg granulomas appear to ac-

tively convert arginine to proline through two intermediates, ornithine and P5C.

Conversion of arginine to proline has been previously demonstrated in the liver slices prepared from *S. mansoni*-infected mice (Dunn *et al.*, 1978b) and in chick embryo legs (Zinker and Rojkind, 1972). Conversely, proline production from glutamic acid has been also found in normal (Sallach *et al.*, 1951; Shen and Strecker, 1975) and injured livers induced with CCl_4 (Rojkind and Diaz de Leon, 1970) or ethanol (Häkkinen and Kulonen, 1975). In the present study, the egg granulomas incubated with ^{14}C-arginine or ^{14}C-glutamate in the medium containing only 0.1 mM each of the respective amino acids have the same results as the egg granulomas incubated with 0.1 mM of both amino acids. Thus, arginine is considered to be a main precursor for proline formation by the egg granulomas.

Dunn *et al.* (1978b) have demonstrated an increased proline synthesis in the liver slices prepared from *S. mansoni*-infected mice. Moreover, because the enzymatic activities involved in arginine-derived proline formation in the egg granulomas were much lower than those of normal and fibrotic livers (Dunn *et al.*, 1981), they suggested a limited capacity of *de novo* proline synthesis by the granulomas, and thus concluded that the bulk of proline available for collagen synthesis is supplied from outside the granulomas. In contrast, we suggested a participation of the egg granulomas in the elevation of hepatic free proline level in murine schistosomiasis mansoni (Tanabe *et al.*, 1991), because the hepatic pool of free proline increases transiently during the 7th to 11th week of infection when host granulomatous reaction is most prominent.

The synthetic rate of arginine-derived proline by the egg granulomas was approximately 1.2 μmoles per g of protein for 6 hr incubation, which is equivalent to 125% and 65% of the proline-synthetic activities measured with the liver slices prepared from uninfected controls and *S. mansoni*-infected mice (Dunn *et al.*, 1978b), respectively. Moreover, this value is almost 2-times higher than the rate of incorporation of radiolabelled proline into TCA-insoluble fraction during *in vitro* incubation of the liver slices prepared from the infected mice in the presence of 0.1 mM proline (Dunn *et al.*, 1978b; Tanabe and Kaneko, *unpublished observations*). It is, therefore, likely that the rate of proline synthesis by the egg granulomas may be sufficient to support increased collagen production in the fibrotic liver.

Proline production by the egg granulomas seems to be dynamically controlled by relative activity of proline formation and degradation. The present study did not provide concrete evidence on the mechanism responsible for active conversion of arginine to proline by the granulomas. Moreover, elevation of hepatic pools of free arginine and ornithine, which can be substrate for proline synthesis, was not found in *S. mansoni*-infected mice (Tanabe and Kaneko, *unpublished observations*). In contrast, the activity of proline-degrading enzymes was low in the egg granulomas, as is also demonstrated in experimental liver cirrhosis (Ehrinpreis *et al.*, 1980; Baich and Chen, 1979), which may contribute to active proline synthesis by the granulomas. Moreover, it is shown in Fig. 6 that proline-forming and -degrading pathways proceed through the same cyclic intermediate, P5C. It seems, therefore, interesting that rates of glutamate formation from arginine or proline by the granulomas were much lower than that of arginine-derived proline formation, because analysis of apparent Km values for substrate and coenzymes of pyrroline-5-carboxylate reductase and glutamic-γ-semialdehyde dehydrogenase indicates that glutamate formation from P5C appears to be predominate pathway in the egg granulomas (Tanabe and Kaneko, *unpublished observation*). This find-

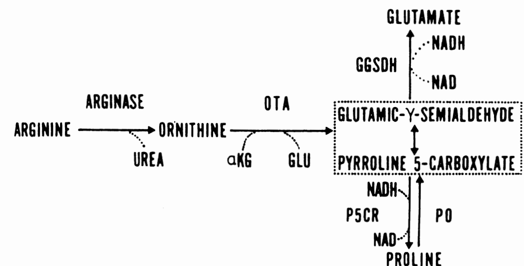

Fig. 6 Presumptive metabolic pathways of proline biosynthesis from arginine and of proline degradation to glutamic acid in *Schistosoma mansoni* egg granulomas. Abbreviations were used as follows: Glutamic-γ-semialdehyde dehydrogenase (GGSDH); Pyrroline-5-carboxylate reductase (P5CR); Ornithine-δ-transaminase (OTA); Proline oxidase (PO); α-Ketoglutaric acid (αKG); Glutamic acid (GLU).

ing may suggest another possibility that metabolic characteristics of the egg granulomas, for example relative concentration of NADH and NAD^+, cause an increase of their arginine-derived proline formation, as is postulated in experimentally induced granulation tissue (Aalto *et al.*, 1973) and in rat liver with ethanol administration (Häkkinen and Kulonen, 1980; Veech *et al.*, 1972).

Elevation of free proline pool has been observed in the fibrotic livers of human (Dunn *et al.*, 1979) and mice (Dunn *et al.*, 1978b; Tanabe *et al.*, 1991) with schistosomiasis mansoni. The supply of free proline is considered to be an important determinant of the rate of hepatic collagen synthesis in schistosomiasis (Dunn *et al.*, 1977) as well as in the liver fibrosis induced by other causes (Henley *et al.*, 1977; Kershenobich *et al.*, 1970; Rojkind and Diaz de Leon, 1970). Since an active collagen synthesis has been demonstrated in the egg granulomas (Dunn *et al.*, 1977; Mereza *et al.*, 1989), the present data suggest that conversion of arginine to proline by the egg granulomas may have patho-physiological significance in the development of liver fibrosis in schistosomiasis mansoni.

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