BIOCHEMICAL STUDIES IN AVIAN MALARIA (*P. GALLINACEUM*)

Effect of *P. gallinaceum* Infection on Amino Acids of the Chick

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ABSTRACT

An analysis of the free and combined amino acids of the whole blood, plasma, erythrocytes, liver and brain of chicks infected with *Plasmodium gallinaceum*. broadly indicate four patterns of reaction.

The salient features of the changes noticed are:

(*a*) A steady increase of almost all the amino acids (free and combined) in the erythrocytes of the infected chick both during the prepatent period and the peak of parasitæmia.

(*b*) A gradual decrease of the amino acids free and combined in the plasma during both the phases of infection.

(*c*) An increase of the free and diminution of certain of the bound amino acids in the whole blood during the peak of parasitæmia.

(*d*) The combined amino acid content of the liver and brain tissues show a steady decrease of almost all the amino acids during both the periods of infection.

Six to eight fold increase of certain amino acids in the erythrocytes during the parasitæmic stage stresses the desirability for a detailed investigation of the effects of these individual amino acids and their specific antimetabolites on the malarial infection.

Significant therapeutic advances and the general and specific preventive measures have resulted in the reduction of the mortality and morbidity from malaria and as such the problems relating to the fundamental nature of the parasite and of the host reactions have yet not been fully investigated even though these studies provide an invaluable basis for the long range control of the disease and enhance the opportunity for the discovery of new and better chemotherapeutic agents.

The pathology of malarial infection has been frequently described, but little information exists concerning the mechanism by which the parasite exerts a damaging effect on its host. The exo-erythrocytic cycle of development of the parasite wherein the parasite grows and develops in the various tissues of the host during the incubation period and the later histopathological lesions of the organs, would exert an influence on the protein and amino acid metabolism of the host. The

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intracellular location of the parasite in the red blood cells of the host and the utilisation of the hæmoglobin for its rapid multiplication also would induce a considerable effect on the protein composition of the blood. Besides the protein variations in the host, increasing recognition is now being given to the role of the changes in the amino acid composition of the blood and tissues during the course of various diseases, in the causation of the severity, in the manifestations of various clinical syndromes, like asthemia and mental disorders[1] and as also other systemic affections.[2] Increase in the blood level of threonine in guinea pigs infected with *S. typhinurium*,[3] causes marked reduction in the survival time. This amino acid, *in vivo*, has been demonstrated[4] to induce mutants of increased virulence. The role of amino acids, not merely as nutritional elements required both for the host and the parasite, but as causal factors exhibiting pharmacodynamic effects resulting in the symptoms of the disease and in ameliorating or enhancing the severity of the illness are facets of enquiry which have not yet had the deserved attention.

The study reported here was undertaken to determine the changes that occur in the free and bound amino acids of the blood, liver and brain of the chicks infected with *P. gallinaceum*. These tissues were chosen because of the intimate relationship of the malarial parasite with these organs of the host. The development of the exo-erythrocytic cycle in the brain of the chick is shown in Fig. 1. The entire erythrocytic development takes place in the blood. The liver has also been shown to be considerably affected during the disease.[5] Hence the variations noted in these tissues would assist in shedding light on some aspects of host-parasite relationship and indirectly might help in explaining some of the complex symptoms of the disease.

EXPERIMENTAL

Young chicken of 4 to 6 weeks old, hatched and bred under standard laboratory conditions and belonging to the white leghorn of Rhode Island type, were used for investigations. The strain of *P. gallinaceum* got from Southern Branch of Malaria Institute of India, Coonoor, and maintained by weekly transfers in the laboratory was used for infections.

Experimental birds were divided into groups of three and kept in individual cages. They were fed once in the mornings with the laboratory diet and blood samples were drawn on the next morning before giving any diet thus allowing a period of about 24 hrs. to eliminate the effects due to amino acids of the diet during the process of absorption. 2 ml. of blood was drawn by cardiac puncture from each bird into a citrated centrifuge tube (10 mgm./ml. of blood) and was used for the analysis of amino acids.

Details of the method of infection and estimation of the degree of parasitæmia have been described earlier.[6] The samples similar to the normals were taken from the chicks during the incubation period and at the height of parasitæmia for successive analysis.

Preparation of samples for the estimation of amino acids.—The procedure adopted for the preparation of the samples for the estimation of the free and combined amino acids from whole blood, plasma and erythrocyte was similar to that reported by Awapora *et al.*[7] and in brief was as follows:—

Whole blood.—0·2 ml. of whole blood treated with 0·6 ml. of ethyl alcohol was centrifuged to facilitate separation of precipitated proteins. Alcoholic layer mixed with three volumes of distilled chloroform was centrifuged and the well separated top layer was pipetted out and the volume of the extract noted.

The protein separated was hydrolysed by a modified method of Dustin *et al.*[8] in a conical flask with 50 ml. of 5 NHCl by autoclaving for 6 hrs. at 15 lb. pressure. HCl was removed by repeated vacuum distillations and the residue taken up in distilled water, neutralized and the volume was made up to 5 ml.

Erythrocytes.—Volume of erythrocytes obtained by centrifugation was washed with saline and the red cell volume was made up to 0·5 ml. This was ground with glass powder with 1·5 ml. of alcohol, centrifuged and the extract was treated as in the case of whole blood and the free amino acids. The residue was hydrolysed with 50 ml. of 5 N HCl as above.

Plasma.—0·2 ml. of plasma was treated in the same manner as in the case of blood and erythrocytes to get the free as well as the combined amino acids.

Liver and brain.—One gram of each of the samples (wet weight) mixed with 1 ml. of water was homogenised with 6 ml. of ethyl alcohol. The homogenate was centrifuged and alcoholic extract was treated with thrice the volume of chloroform to get the free amino acids. The residue was dried overnight below 105° C. and 20 mgm. of dried and pulvarised powder was refluxed with 150 ml. of 5 N HCl for 20 hrs. as per the procedure of Dustin *et al.*[8] and after the removal of HCl, the neutralised hydrolysate obtained was subjected to amino acid analysis.

Method of estimation of amino acids.—Amino acids present in the above hydrolysates were estimated by the circular paper chromatographic method as reported by Rao *et al.*[9] using three chromatograms developed with three different solvent mixtures for each of the hydrolysates. Coloured bands got after ninhydrin spray were identified in preliminary experiment, cut out and extracted with 75% alcohol and the individual amino acid concentrations were calculated by the procedure reported by Giri *et al.*[10] The values of amino acids were expressed as percentage[9] of the total nitrogen by adopting the procedure of Johnson *et al.*[11] for micronitrogen estimation. Results of these experiments are presented in Table I.

Free amino acids.—The method of estimation was similar in all respects to the methods used for the acid hydrolysates. The changes of free amino acids on equal volume basis in the case of blood, plasma and erythrocytes are represented in Table II.

TABLE I

Changes in the concentrations of the amino acids of the whole blood, plasma, erythrocytes, liver and brain of chicken during the course of infection with P. gallinaceum

The results are expressed in percentage of total Nitrogen

Amino acids	Whole Blood			Plasma			Erythyrocytes			Liver			Brain		
	Normal	Incubation Period	Acute infection	Normal	Incubation Period	Acute infection	Normal	Incubation Period	Acute infection	Normal	Incubation Period	Acute infection	Normal	Incubation Period	Acute infection
1. Leucine ..	4·4	2·4	2·7	1·1	1·9	1·4	2·3	3·7	6·7	4·7	4·2	1·6	6·1	5·9	1·5
2. Isoleucine ..	1·6	1·2	1·8	0·6	0·1	1·1	2·0	1·7	3·4	3·0	2·5	1·1	4·1	4·4	1·3
3. Phenylalanine ..	7·1	2·2	2·7	0·7	1·6	0·8	1·7	3·0	5·5	3·9	4·1	1·1	6·9	5·0	1·4
4. Valine ..	2·4	1·4	1·7	0·6	2·0	2·2	0·9	2·9	3·6	3·5	2·4	1·0	..	..	..
5. Methionine ..	1·0	0·6	1·3	0·5	0·3	0·1	0·3	0·7	1·4	0·9	0·7	0·3	..	..	..
6. Tyrosine ..	1·1	1·1	1·0	0·4	0·4	0·4	0·7	1·3	1·5	2·1	0·6	0·2	2·7	0·3	0·3
7. Alanine ..	4·6	2·6	3·3	1·7	2·0	1·5	2·0	5·1	6·2	4·1	3·3	1·6	5·1	4·8	2·0
8. Threonine ..	2·3	1·4	2·0	0·9	2·0	1·5	1·2	2·3	4·3	3·0	2·7	1·2	2·5	1·5	2·5
9. Glutamic acid ..	3·1	1·7	2·5	1·7	2·7	1·5	1·6	3·0	5·5	1·1	2·0	2·0	0·5	1·6	1·0
10. Glycine ..	2·8	1·5	2·8	2·8	1·0	1·3	1·4	2·5	6·0	4·1	4·2	3·3	5·7	2·7	1·5
11. Serine ..	2·3	1·1	2·2	0·9	2·0	1·3	1·0	1·3	2·5	1·6	1·5	1·4	2·4	1·1	1·4
12. Aspartic acid ..	4·9	2·9	3·0	2·3	4·0	2·8	0·8	2·6	7·8	6·0	4·5	2·4	6·5	5·5	0·4
13. Arginine ..	20·3	12·5	13·7	10·2	17·0	11·3	3·4	9·0	7·4	9·6	8·0	3·7	18·8	29·5	10·0
14. Histidine ..	2·3	2·4	0·9	..	..	..	0·3	0·6	1·0	2·9	2·7	1·5	3·8	4·4	2·0
15. Lysine and ornithine	11·7	5·8	5·0	1·4	3·1	1·4	1·3	3·7	4·1	12·4	12·0	5·2	1·6	1·4	2·6
16. Cystine ..	3·7	1·3	0·4	0·8	3·5	2·1	1·2	1·5	2·8	1·6	3·8	3·0	9·0	5·2	2·1

TABLE II

Summary of the changes in the amino acids (free) of the whole blood, plasma, erythrocytes, liver and brain of chick en during the course of infection (P. gallinaceum)

	Amino Acids			
	Decreased	Increased	Decreased during prepatent and increased in parasitæmic stage	Increased during prepatent and decreased in parasitæmic stage
Whole blood ..	Peptide (Below cystine) band	Leu.; Isoleu.; Ph. al.; Val.; Meth.; Tyro.; Ala.; Thre.; Gl. acid; Gly.; Serine; Arg.; Lys.; Cyst.	Aspartic acid, Histidine	..
Plasma ..	Leu.; Gl. acid; Serine; Arg.	..	Tyr.; Ala.	Isoleu.; Ph. al.; Val.; Meth; Thr.; Asp. acid; His.; Lys.; Cyst.
Erythrocytes ..	Hist.	Ser.; Asp.; Arg.	Leu.; Isoleu.; Ph. al.; Val.; Meth.; Tyr.; Ala.; Thr.; Gl. acid; Gly.; Hist.; and Peptide (below cystine)	Cystine
Brain ..	Isoleu.; Ph. al.; Tyr.; Gl. acid; Gly.; ser.; Asp. acid; Lys.	..	Leu.; Val.; Meth.; Ala.; Thr.; Arg.; Cyst.; peptide (below cystine)	His.
Liver ..	Ph. al.; Tyr.	Ala.; Lys.;	Leu.; Isoleu.; Val.; Meth.; Thr.; Gl. acid; Gly.; Ser.; Arg.; His.; Cyst; peptide	Asp. acid

Note.—Leu., leucine; Isoleu., isoleucine; Ph.al., phenylalanine; Val., valine; Meth., methionine; Tyr., tyrosine; Ala., alanine; Thr., threonine; Glut. acid, glutamic acid; Gly., glycine; Ser., serine; Arg., arginine; Lys., lysine; Cyst., cystine; Asp. acid., aspartic acid; His., histidine.

DISCUSSION

An analysis of the amino acid changes (free and combined) in the blood and tissues during malarial infection indicate in general the following four patterns of reaction.

1. A continuous decrease of certain amino acids during the entire period of infection.

2. A continuous increase of certain amino acids during the entire period of infection.

3. An initial increase during the prepatent period and diminution in the parasitæmic stage and lastly,

4. An initial decline followed by an increase.

From Table I it is clear that in the hydrolysate of the whole blood leucine, phenylalanine, valine, methionine, glycine, lysine and cystine are reduced by nearly 50% during the incubation period whereas in acute stage isoleucine and methionine alone reach higher levels than the initial concentration even though all the amino acids also show a tendency to increase.

Many of the amino acids like cystine, valine, alanine, phenylalanine, threonine, leucine, glutamic acid, lysine and arginine increase slightly in the plasma during incubation period followed by a fall during the acute stage.

Erythrocytes on the other hand present a different picture. All the amino acids steadily increase during both the incubation period and peak of parasitæmia. Aspartic acid, valine, glycine, threonine and phenylalanine are considerably increased during the height of infection from 3 to 6 times the original value while the rest of the amino acids show an increase of two to three times the normal.

Liver and brain tissues show a steady decrease of all the amino acids from the period of infection excepting cystine which alone is increased in liver.

In contrast to the pattern of bound amino acids almost all the free amino acids of whole blood increase during the period of infection, but register a fall in case of plasma. The free amino acids of erythrocytes excepting cystine and histidine increase in the parasitæmic stage.

Phenylalanine, tyrosine and aspartic acid were the free amino acids which decrease in the liver with the progress of infection whereas all other amino acids decrease in the incubation period and then increase in the parasitæmic stage. From Table II it is also clear that there is a decrease of a majority of free amino acids in the brain tissue.

The values of the amino acids in blood and tissues are the resultants of various factors like the changes in the proteins, the diminution in the number of erythrocytes, the presence of the proteins of the parasite and antibodies and the influence of infection on functional capacity of organs like liver which has prominent role in protein metabolism.

The diminution of both free and combined amino acids in plasma is a significant observation. The interference with protein metabolism as reflected by histopathological lesions of the liver and biochemical analysis of the proteins of blood, the utilisation of some of the amino acids by the host for its nutrition and for the antibodies formation and finally the increased demand made by the parasite for its growth and rapid multiplication are probably the contributory causes for lowering of amino acid content of plasma.

The increase of both the forms of amino acids in the erythrocytes is due to the rapid hydrolysis of the globin fraction of hæmoglobin by the parasites, to amino acid components and to the presence of parasites in erythrocytes. Changes in free amino acids of liver and brain as shown in Table II do not follow the same pattern in both tissues.

Though no definite conclusions can be drawn from these observations regarding the *in vivo* nutritional requirements of the malarial parasites the very high increase of certain amino acids in the erythrocytes is of great importance. Further studies on the influence of these individual amino acids and their specific antimetabolites on experimental infection might contribute to a clearer understanding of the role of these amino acids in the nutritional needs of the malarial parasite.

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REFERENCES

1. Weil-Maltrebe, H. .. *Physiol. Rev.*, 1950, **30**, 549.
2. Sirsi, M. .. Unpublished observation on the Pharmacodynamic effects of amino acids.
3. Holtman, D. F. and Page, L. A. Quoted from *J. Bact.*, 1955, **70**, 272.
4. Page, L. A., Goodlow, R. J. and Brown, W. *J. Bact.*, 1951, **62**, 639.
5. Ramaswamy, A. S., Rama Rao, R. and Sirsi, M. *J. Ind. Inst. Sci.*, 1955, **37**(1), 44.
6. Rama Rao, R., Viswanathan, K. V., Ramaswamy, A. S. and Sirsi, M. *Curr. Sci.*, 1953, **22**, 305.
7. Awapora *et al.* .. *Paper Chromatography*, by R. J. Block, Academic Press, New York, 1952.
8. Dustin, J. P., Schram, E., Moore, S. and Bigwood, E. J. *Bull. Soc. Chim. Biol., Paris*, 1953, **35**, 1137.

9. Rao, N. A. N. and Wadhwani, T. K. — *J. Ind. Inst. Sci.*, 1955, **37**, 130.

10. Giri, K. V. and Rao, N. A. N. — *Ibid.*, 1953, **35**, 145.

11. Johnson, M. .. *J. Biol. Chem.*, 1941, **137**, 576.

12. Almquist, H. J. .. *J. Nutrition*, 1947, **34**, 543.

13. Geurian, Q. M. and McKee, R. W. — *J. Parasit.*, 1950, **36**, 211.

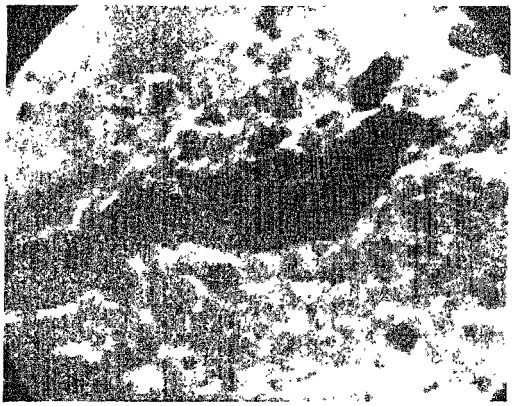

FIG. 1. Smear from the brain showing the presence of Exoerythrocytic form of *P. gallinaceum*.