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Cofactors and vitamins in the metabolism of malarial parasites. Factors other than folates

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Relatively few cofactors have so far been demonstrated to be essential for the intracellular development of erythrocytic stages of malarial parasites. Besides 4-aminobenzoic acid, presumably required for the synthesis of folates, these are biotin and pantothenate. The pantothenate is not used directly by the parasites but rather as coenzyme A synthesized by the host erythrocyte. Parasites maintained extracellularly in vitro *also have a requirement for exogenous adenosine triphosphate. No information is available concerning cofactor requirements of the sporogonic or pre-erythrocytic stages.*

The erythrocytic stages of malarial plasmodia are obligate intracellular parasites. Until very recently (*1*) none of them could be grown beyond one or a few cycles of schizogony away from an intact living host. Accordingly, most observations on their requirements for cofactors, as well as other nutrients, have been of a more or less indirect nature. Thus Maier & Riley (*2*) found that the antimalarial effect of sulfonamides against erythrocytic stages of *Plasmodium gallinaceum* in chicks was completely prevented by simultaneous administration of 4-aminobenzoic acid. This indicated that 4-aminobenzoic acid is an essential cofactor for malarial parasites, an indication later confirmed and extended by Ball et al. (*3*) for the monkey malaria *P. knowlesi* developing in red-cell suspensions *in vitro* and for the rodent malaria *P. berghei* in intact rats (*4*). Since 4-aminobenzoic acid enters into the synthesis of folates, its role will be considered in more detail by Dr Ferone (see pages 291-298).

In a study of the influence of biotin deficiency on the course of avian malaria infection, Trager (*5*, *6*) noted that the deficiency always increased parasitaemia in chicks and ducks infected with *P. lophurae*, indicating an effect mainly on the innate resistance of the hosts. With *P. cathemerium* in ducks, the results were more complex. With very severe deficiency of biotin, multiplication of the parasites was only half that found in the controls, but the hosts were approaching death from the nutritional deficiency itself so that other factors may well have played a role. In moderately deficient ducks, *P. cathemerium* at first multiplied more slowly than in controls fed a diet adequate in biotin. Later, however, higher parasitaemia developed in the deficient birds. These results suggest two interacting effects, one from a requirement for biotin by the parasites, the other from the need for biotin for resistance of the host. Siddiqui et al. (*7*) later showed that biotin favours the early *in vitro* development of *P. knowlesi* in monkey erythrocytes (Table 1). It seems reasonable to conclude that biotin is among the cofactors essential to the intraerythrocytic development of plasmodia. Indications are that it is required at concentrations lower than those at which the host would be adversely affected. Hence analogues of biotin are not likely to be suitable candidates for anti-malarial drugs.

Riboflavin deficiency in chickens (*8*) and vitamin C deficiency in monkeys (*9*) inhibited multiplication of malarial parasites (*P. lophurae* and *P. knowlesi*, respectively). These results may indicate that both these vitamins are essential to the parasites, but no *in vitro* effects of their omission have been noted (see Table 1). In view of the effect of riboflavin in raising the levels of glutathione reductase (NAD(P)H) (EC 1.6.4.2) in erythrocytes (*10*, *11*), and in view of the importance of glutathione in the extracellular survival of *P. lophurae* (Trager, unpublished data), it could well be that riboflavin plays such an indirect, as well as a direct, role in malarial metabolism.

Much more is known about the role of pantothenate. The effect of this vitamin was first noted in experiments with *P. lophurae* in duck erythrocytes maintained *in vitro* (*12*). When calcium pantothenate was added to a complex medium at levels of 0.02–

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Table 1. Effect of deletion of growth factors on development of *Plasmodium knowlesi* in erythrocyte suspension *in vitro* [a]

Factors omitted	Ratio of parasites per 100 red cells at 24 h to that at start
none	2.5
ascorbic acid	2.6
4-aminobenzoic acid	2.2
riboflavin	2.4
biotin	1.4
thiamin, niacin, nicotinamide, cocarboxylase, D-Ca pantothenate, pyridoxine, ribose, and choline	2.45

[a] From Siddiqui et al. (7).

0.03 mg/ml, infectivity was demonstrated after 8 days *in vitro* and the presence of viable male gametocytes capable of exflagellation after up to 16 days. In the absence of the added pantothenate, both infectivity and exflagellation persisted only about 5 days. This result was soon confirmed. In experiments with chickens infected with *P. gallinaceum*, either dietary pantothenate deficiency, or administration of an antimetabolite of pantothenate, markedly inhibited development of the parasites (*13*). In short term *in vitro* experiments with the human malarial parasite *P. falciparum* and the monkey malaria *P. coatneyi* it wrs shown (*14*, *16*) that antimetabolites of pantothenate inhibited development (Tables 2 and 3). Pantothenate would seem to be an essential cofactor for erythrocytic malarial parasites.

Table 2. Effects of antipantothenates on *P. coatneyi* after 2 days *in vitro* [a]

Flask No.	Anti-pantothenate	Parasites per 10 000 erythrocytes	
		Normal	Abnormal
1	none	56	0
2	none	72	0
5	WR54036 25 μg/ml	3	1
6	WR54036 25 μg/ml	3	1
9	SN14622 75 μg/ml	7	2
10	SN14622 75 μg/ml	2	3

[a] From Trager (*15*). This paper must be seen for details.

Table 3. Effects of antipantothenates and of primaquine on *P. falciparum* (in *Aotus* monkey erythrocytes) after 2 days *in vitro* [a]

Flask No.	Anti-pantothenate	Conc. (μg/ml)	Parasites per 10 000 erythrocytes	
			Normal	Abnormal
1	None		25	2
2	None		23	5
3	SN 14622	150	5	8
4	SN 14622	150	2	12
5	WR 54036	75	1	8
6	WR 54036	75	1	8
7	WR 54036	50	2	6
8	WR 54036	50	0	10
9	WR 54036	25	9	10
10	WR 54036	25	7	12
11	primaquine	20	13	12
12	primaquine	20	6	13

[a] From Trager (*15*). This paper must be seen for details.

The pantothenate is not used directly, however. In studies with *P. lophurae*, the one species of malarial parasite that has been kept alive and developing extracellularly *in vitro* for several days (*16*, *17*), it was soon found that coenzyme A (CoA) but not pantothenate had a favourable effect on the extracellular development (*18*, *19*). Furthermore, precursors of CoA, like pantothenate, were ineffective (*20*) (Tables 4 and 5). CoA could be replaced by dephospho-CoA, but this was probably a result of enzymatic synthesis of CoA by the erythrocyte extract of the culture medium. None of the enzymes of CoA biosynthesis could be found in erythrocyte-free *P. lophurae* although all of them could be readily demonstrated in both normal and *P. lophurae*-infected duck erythrocytes (*21*, *22*). These enzymes were: pantothenate kinase (EC 2.7.1.33); phosphopantothenoyl-cysteine synthetase (EC 6.3.2.5); pantothenoyl-cysteine decarboxylase (EC 4.1.1.30); dephospho-CoA pyrophosphorylase (pantetheine-phosphate adenylyltransferase, EC 2.7.7.3); and dephospho-CoA kinase (EC 2.7.1.24). It was noteworthy that the activity of all of these enzymes was decreased in erythrocytes containing large parasites. The conclusion seems inescapable that malarial parasites (or at least *P. lophurae*, the one

Table 4. Ineffectiveness of phosphopantothenic acid (PPA) as replacement for coenzyme A (CoA) in the extracellular development *in vitro* of *Plasmodium lophurae* [a]

Expt.	Flask	CoA (mmol/litre)	PPA (mmol/litre)	Mean percentage of parasites after 20 h		cpm/100 × 10^6 parasites per h of exposure [b]
				with > 1 nucleus	Degenerate	
A	1–3	0	0	16	9	398
	4–6	0	0.06	15	7	414
	7–9	0.05	0	21	4	665
B	1–3	0	0	12	16	207
	4–6	0	0.06	16	22	266
	7–9	0.05	0	16	7	356

[a] From Trager & Brohn (*20*). Medium was one-third strength erythrocyte extract (4% duck haemoglobin).

[b] Incorporation of L-methionine-methyl-^{14}C added at 16 h.

Table 5. Partial replacement of coenzyme A (CoA) by dephospho-CoA (d-CoA) and phosphopantotheine (PPS), but not by phosphopantothenylcysteine (PPC), in the extracellular development *in vitro* of *Plasmodium lophurae* [a]

Expt.	Flask	Supplement (all at 0.05 mol/litre)	Mean percentage of parasites after 20 h		cpm/100 × 10^6 parasites per h of exposure
			with < 1 nucleus	Degenerate	
A	1–4	None	27	7	179
	7–8	CoA	29	3	259
	9–12	d-CoA	29	4	263
B	1–3	CoA	30	11	646
	4–6	PPC	16	16	473
	7–9	PPS	21	13	602
	10–12	None	15	16	347

[a] From Trager & Brohn (*20*). Medium was one-third strength erythrocyte extract (4% duck haemoglobin).

[b] Incorporation of L-methionine-methyl-^{14}C in Expt. A, of L-isoleucine-U-^{14}C in Expt. B, added at 16 h.

species with which appropriate studies have been done) have in their erythrocytic stage a major biosynthetic lesion. For their CoA, a material essential to their metabolism (*23*), they depend completely on the biosynthetic activity of their host erythrocytes.

It is obvious that these studies must be extended to other species of malarial parasites. With the continuous cultivation of *P. falciparum* (*1*) and with the preliminary observations on the short-term extracellular maintenance of this species (*24*) the way is open for such experiments with this important parasite. It would also be of great interest to discover whether other analogous biosynthetic lesions exist in erythrocytic malaria parasites. Pyridoxine kinase (EC 2.7.1.35) would seem a likely candidate especially in view of the much lower activity of this enzyme in the red cells of Africans than in those of whites (*25*). If the enzyme is lacking in the parasites, its presence

at a lower level in the host erythrocytes might have a favourable selective effect in regions where malaria is endemic. Similarly, it would be of interest to compare levels of pantothenate kinase in erythrocytes of West African Negroes and in Caucasians. A large proportion of peopie with β-thalassaemia (a trait that may be selected for by malaria (*26*)) also have, as an independently inherited character, an abnormally slow rate of conversion of pyridoxine to pyridoxal phosphate in their red cells (*27*).

The work with CoA indicates two equally valid approaches to the further study of cofactors in the metabolism of malarial parasites: (1) comparative measurements of biosynthetic enzyme activities in infected and normal erythrocytes and in the free parasites; (2) direct nutritional experiments with parasites maintained extracellularly *in vitro*. Experiments of the former type should be possible with any species of parasite available in the laboratory. Experiments of the latter type, done so far only with *P. lophurae*, are hampered by the need for erythrocyte extract in the medium, thereby presumably supplying a variety of cofactors of undefined nature. Nevertheless, in these experiments it was possible to show not only the CoA requirement, but also requirements for glutathione and for a high level of nicotinamide (Trager, unpublished data). Furthermore, the axenic parasites have a striking requirement for an exogenous source of adenosine triphosphate (ATP). Since *P. lophurae* does have its own enzymes for the two steps in the formation of ATP in the glycolytic cycle (pyruvate kinase, EC 2.7.1.40, and phosphoglycerate kinase, EC 2.7.2.3 (*28*)) it seems possible that the ATP is essential only for the functioning of the outer, originally host-cell derived, membrane that surrounds the parasite plasma membrane and is closely apposed to it during growth of the trophozoite (*29*). In keeping with this hypothesis, are the effects of bongkrekic acid, an inhibitor of mitochondrial ATPase and cation-transport (*30*). On the other hand, it is equally possible that ATP as such is actually taken up by the parasites. That ATP can in fact enter cells has now been amply demonstrated (*31*).

Little is known of the nutritional requirements of the two developmental cycles of malarial parasites other than the erythrocytic cycle. Avian exoerythrocytic forms can be grown continuously in tissue culture (*32*) but such cultures have not been used in attempts to study cofactors for the parasites. The sporogonic stages of avian malaria developing in *Aedes aegypti* mosquitos were shown by Terzian (*33*) to be affected by a wide range of metabolites and antimetabolites, but the results were not brought to the stage where particular requirements could be established. A vast field remains to be investigated.

RÉSUMÉ

COFACTEURS ET VITAMINES DANS LE MÉTABOLISME DES PARASITES DU PALUDISME: FACTEURS AUTRES QUE LES FOLATES

Relativement rares sont les cofacteurs dont il a jusqu'ici été possible d'établir le caractère indispensable au développement intracellulaire des stades érythrocytaires des parasites du paludisme. Outre l'acide 4-aminobenzoïque, vraisemblablement nécessaire à la synthèse des folates, ces cofacteurs sont la biotine et le pantothénate. Ce dernier n'est pas utilisé directement par les parasites mais plutôt comme coenzyme A synthétisé par l'érythrocyte hôte. Les parasites conservés *in vitro* en dehors des cellules ont également besoin d'adénosine triphosphate exogène. On ne possède aucun renseignement concernant les besoins en cofacteurs aux stades sporogonique et pré-érythrocytaire.

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