The Sulphur Requirements of the Erythrocytic Form of *Plasmodium knowlesi*

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Until the discovery by Knowles & Das Gupta (1932) of a malaria parasite virulent for rhesus monkeys, the study of mammalian malaria in the laboratory was seriously hampered. This parasite, which is mildly infective to man, was named *Plasmodium knowlesi* by Sinton & Mulligan (1932).

Although extensive studies have been made on the culture and metabolism of the erythrocytic form of *P. knowlesi* (for reviews see McKee, 1951 and Fulton, 1951), little is known about the extent to which the parasite can satisfy its nutritional requirements from the cytoplasmic content of its host cell, the erythrocyte. The growth of the parasite in the erythrocyte is associated with a reduction in the haemoglobin content and the formation of parasite pigment. This pigment has been identified as haematin (Morrison & Anderson, 1942; Rimington & Fulton, 1947) and is derived from the haem moiety of globin, the total pigment (haem plus haematin) content of the parasitized cell remaining constant (Ball, McKee, Anfinsen, Cruz & Geiman, 1948). In contrast, the total nitrogen of the infected erythrocyte decreases, and this loss has been attributed to the hydrolysis of globin by the parasite and the utilization of about half the products of hydrolysis for the synthesis of its own protein (Morrison & Jesky, 1948; see also McKee, 1951). In spite of this apparent excess of available amino acids, an additional source of methionine appears to be essential for the growth of *P. knowlesi* both *in vitro* and *in vivo* (McKee, Geiman & Cobbey, 1947; Geiman & McKee, 1948). The reasons for this specific requirement are not known, but it has been suggested that the low methionine content of haemoglobin may make it necessary for the parasite to draw on the plasma for additional supplies of this amino acid (McKee, 1951).

The present paper is concerned with the sulphur-containing compounds within the monkey erythrocyte, and their utilization by the parasite for the synthesis of its own protein.

EXPERIMENTAL

Source of plasmodia. The strain of *P. knowlesi* used was obtained from Dr D. G. Davey of I.C.I. Pharmaceuticals Ltd., Wilmslow, Cheshire. It had recently been isolated in Malaya and proved very virulent for *Macacus rhesus* hosts in which it was passaged. A chronic infection in this host was maintained by the method of Edeson & Davey (1953), with atebrin. The development cycle is of 24 hr. duration and most of the parasites are at the same stage of growth at any one time (synchronous infection).

Cell counts. Total red-cell counts were made in a Thoma–Zeiss haemocytometer. The percentage of parasitized cells was determined in preparations stained by the Leishman method. Parasites freed from host cell by treatment with saponin were also counted in the haemocytometer. The percentage of reticulocytes was determined in a fresh red-cell suspension stained supravitally with 1% (w/v) Brilliant Cresyl Blue in 0·6% (w/v) saline.

Labelled compounds. DL-[^{35}S]Methionine was obtained from the Radiochemical Centre, Amersham. DL-[^{35}S]-Cystine was synthesized as described by Arnstein & Grant (1954). DL-[^{35}S]Cysteine hydrochloride was prepared from DL-[^{35}S]cystine as described by Lucas & Beveridge (1940). Before use, the hydrochloride was converted into the free amino acid by neutralizing an ethanolic solution with pyridine. The precipitate of DL-[^{35}S]cysteine was filtered off and well washed with ether. On titration with 0·01 N iodine, this sample required 97% of the theoretical amount calculated for the free thiol compound.

In vitro *incubation.* (*A*) Monkey blood (100 ml.) containing 18% infected cells (all half-grown parasites) was obtained by cardiac puncture, with heparin added as anticoagulant. This volume of blood was mixed with 20 ml. of a synthetic nutrient medium so that the final concentration of the components (salts, sugars, amino acids, vitamins and nucleic acid bases) in the suspension was that given by Geiman (1949). The blood suspension was dispensed in 10 ml. amounts into 50 ml. conical flasks and rocked (12 rev./min., rocking arc 30°) for 4 hr. at 37°. During this time a slow stream of a water-equilibrated mixture of CO_2 in air (5:95, v/v) was passed over each flask. At the end of the incubation period, the parasite material and haemoglobin were isolated as described below.

(*B*) From monkey blood (20% of erythrocytes infected with half-grown parasites), a concentrated suspension of infected erythrocytes (73% parasitized) was obtained by the procedure described by Geiman, Anfinsen, McKee, Ormsbee & Ball (1946). This suspension was centrifuged at 2000 rev./min., (30 cm. head diameter) for 10 min., the plasma removed and 5 ml. of the sedimented cells resuspended in 40 ml. of a serum-containing nutrient medium (Geiman, 1949), in which the amino acid mixture had been replaced by DL-[^{35}S]cysteine (0·033 m-mole) and non-isotopic DL-methionine (0·033 m-mole). Another 5 ml. of the sedimented cells was resuspended in a similar medium in which DL-[^{35}S]methionine (0·033 m-mole) and non-isotopic DL-cysteine (0·033 m-mole) were present. Each of

these suspensions was incubated at 37° in five flasks containing 8 ml. amounts as described above. At intervals, one flask from each group was removed, the cells were isolated by centrifuging and washed three times in isotonic saline containing 0·2% (w/v) glucose, and non-isotopic cysteine and methionine in the concentrations given above. The washed sedimented cells were then freeze-dried. Crude protein was isolated and oxidized with performic acid as described below.

Production of ^{35}S*-labelled erythrocytes and their transfusion into a monkey parasitized with* Plasmodium knowlesi. The erythrocytes of a small monkey (2¼ kg.) were checked for compatibility with the plasma of a larger one (3¼ kg.). Reticulocytosis was induced in the smaller animal by daily bleeding, a total of 50 ml. being removed in a period of 4 days. At 41 hr. after the final bleeding, when approximately 16% of the total red cells were reticulocytes, a single dose of DL-[^{35}S]methionine (5 mg., 250 μc) was injected intraperitoneally. The incorporation of radioactivity into the red cells was followed and was found to reach a maximum 8 days after the injection of the labelled material.

During this time, the larger monkey was infected with *P. knowlesi*. When approx. 10% of the erythrocytes contained nearly full-grown parasites the smaller monkey was anaesthetized (12 days after injection of the labelled material) and bled by cardiac puncture into a mixture of 2% (w/v) sodium citrate in 1% (w/v) NaCl. The erythrocytes were centrifuged at 2000 rev./min. (30 cm. head diameter) for 10 min. and washed 4 times with 0·9% (w/v) NaCl containing 0·2% (w/v) glucose. The final suspension was centrifuged, the supernatant removed and 25 ml. of the packed erythrocytes were resuspended in an equal volume of the glucose–saline solution. This suspension was slowly and continuously injected over a 30 min. period into a leg vein of the parasitized monkey. During the whole of this period the animal was lightly anaesthetized with ether.

Isolation of malaria parasites and parasite protein. Usually when about 40% of the red cells were infected, the parasitized monkey was anaesthetized with ether and 1 ml. of a 1% (w/v) heparin solution injected intravenously. The animal was bled by cardiac puncture into citrated saline containing 0·2% (w/v) glucose. The vascular system was then perfused with saline by means of a cannula in the aorta and the perfusing liquid collected by a cannula in the inferior vena cava.

The total blood was centrifuged at 2000 rev./min. for 10 min., the diluted plasma was retained and the blood cells were resuspended in an equal volume of fresh citrated saline. This suspension was slowly drawn through columns of dry Solka-Floc (Johnsen, Jörgensen and Wettre, London), 2 g./75 ml. of suspension, with a negative pressure in the receiving vessel. Such a procedure removes practically all leucocytes from the blood.

The parasites were then isolated by lysis of the erythrocytes with saponin as described by Christophers & Fulton (1939). After washing the parasites three times in saline, samples were taken for microscopic examination and the remainder was freeze-dried.

In early experiments, pigment was removed from the parasite by extraction with ethanolic KOH (see Deane, 1945), but subsequently cleaner preparations were obtained by extraction with HCl–acetone mixtures. For example, 200 mg. of parasite material, with a few small glass beads to aid breaking up the organism, was shaken for 30 min. on a Microid shaker (Griffin and Tatlock, London) with three successive amounts (10 ml.) of HCl–acetone (1 ml. of 2N-HCl in 100 ml. of acetone). The residue was extracted with two successive amounts (10 ml.) of 5% (w/v) trichloroacetic acid at 90° for 10 min. Finally the pale buff-coloured solid was washed with ethanol and ether, and dried. Yield, 124 mg.

Isolation of globin and total intracellular protein from lysed erythrocytes. After saponin lysis of the erythrocytes and removal of the malarial parasites, the intracellular proteins of the erythrocytes were obtained from the supernatant in the following way: red-cell membranes were agglutinated by passing CO_2 into the solution for 60 min. (Parpart, 1942) and sedimented by centrifuging at 10 000 rev./min. in an angle centrifuge (about 9000 *g*) for 20 min. The clear red solution which remained was dialysed against two successive amounts (100 vol.) of physiological saline at 0° for 24 hr. For isolation of total protein from the residue in the sac a portion was treated at 90° with trichloroacetic acid for 10 min. at a final concentration of 10% (w/v). The precipitate was centrifuged and re-extracted with 10% (w/v) trichloroacetic acid, washed with ethanol and ether, and dried. Globin was isolated from another portion by the acid–acetone technique of Anson & Mirsky (1929–30).

Purification of isotopic protein samples. Before hydrolysis or direct radioassay of the protein samples, it was necessary to eliminate the possibility of adsorption or attachment by disulphide linkage of free sulphydryl compounds to the protein. For this purpose, each sample (150 mg.) was oxidized in performic acid (10 ml. of 98%, w/v, formic acid and 2 ml. of 100 vol. H_2O_2) and protein in the clear solution was reprecipitated with trichloroacetic acid (see Peterson & Greenberg, 1952). In this procedure, cystine is oxidized to cysteic acid and methionine to its sulphone.

Dilution of labelled protein and the isolation of cystine and methionine. From an infected monkey usually only 100–400 mg. of isotopic parasite protein was obtained. Before hydrolysis, such samples were accurately diluted by weight with non-isotopic protein isolated in an identical manner from mature parasites.

The protein samples (usually about 0·8–1·0 g.) were hydrolysed with 6N-HCl in sealed tubes at 105° for 18 hr. Hydrochloric acid was removed by repeated evaporation *in vacuo* and the residue kept overnight *in vacuo* over solid KOH. The residual solid was taken up in 5 ml. of water and the filtered solution was then applied to a column of Zeo-Karb 225 (Permutit Co., London) in the hydrogen form (750 g./g. of original protein hydrolysed). The column was developed with 1·5N-HCl, the effluent being collected in 7 ml. fractions, and the amino acids were detected by paper chromatography with phenol–NH_3 soln. as developing solvent. Cysteic acid is the first amino acid to be eluted (Arnstein & Grant, 1954), methionine sulphone being eluted after glutamic acid.

The fractions containing cysteic acid were pooled and evaporated to dryness *in vacuo*, those containing methionine sulphone being similarly treated. Each residue was redissolved in distilled water (2 ml.) and inorganic sulphate precipitated by the addition of a solution of 0·22M benzidine dihydrochloride (1 ml.) followed by anhydrous acetone (2 ml.). After 4 hr. at 4°, the small precipitate of benzidine sulphate was filtered off, and the filtrate evaporated to

dryness. Cysteic acid and methionine sulphone were converted into inorganic sulphate by digestion with the reagent described by Pirie (1932). Because of the recorded difficulty in oxidizing methionine with this reagent (Callan & Toennies, 1941) all samples of methionine sulphone were digested for 72 hr. The inorganic sulphate was precipitated with benzidine by a procedure similar to that described by Simpson & Tarver (1950).

Radioactivity measurements. All measurements were carried out with a helium-filled, bell-shaped end-window Geiger–Müller counter, using 'infinite thickness' samples, the substances being mounted on 1 sq.cm. polythene disks (Popják, 1950). All counts were compared with those of a [^{14}C]Perspex reference standard obtained from the Radiochemical Centre, Amersham, and were corrected for decay by direct comparison with a subsidiary ^{35}S standard sample. The standard error of all determinations was $\pm 5\%$. For assay of ^{35}S as benzidine sulphate, the substance was mounted by filtration on filter paper (Whatman no. 50) on 1 sq.cm. stainless-steel perforated disks. The amount of benzidine sulphate present on a disk was determined by titration at 100° against 0·01 N-NaOH with phenol red as an internal indicator. The amount usually present was 4–10 mg., and all radioactivities were corrected to 'infinite thickness' by means of a correction factor. This factor was obtained from a chart made by plotting the observed radioactivities of a standard benzidine sulphate sample mounted in amounts of 2–50 mg. as described above.

Isolation and determination of glutathione. Erythrocyte glutathione was isolated via the cuprous mercaptide, essentially as described by Dimant, Landsberg & London (1955).

For the estimation of glutathione, about 4 ml. of blood was centrifuged and the white-cell layer removed by pipette. The red cells were resuspended in saline to give the original blood volume, a small sample was taken for cell counts and 1 ml. samples were used to estimate glutathione by the method of Hardin, Valentine, Follette & Lawrence (1954).

Total sulphur. This was determined by the gravimetric wet-combustion method (Carius) as described by Niederl & Niederl (1941).

Determination of cystine and methionine. These amino acids were determined directly in globin by the technique of Evans (1945), in which nitric acid is used as a selective oxidizing reagent. The standard error in this estimation is about $\pm 10\%$.

RESULTS

Preparation of malaria parasites essentially free of the host erythrocyte. After saponin lysis of infected erythrocytes and extensive washing of the parasites, stained preparations (Leishman) appear normal and no erythrocytic membrane is visible (cf. Christophers & Fulton, 1939). Ultraviolet microscopy, however, suggests that some of the parasites may still be attached to the erythrocyte membrane, but this was not studied further since calculation showed that the mass of these membranes relative to that of the parasite was insignificant.

Amino acid composition of the parasite protein. No unusual amino acids were detected when hydrolysates of parasite protein were analysed on columns of Zeo-Karb 225. Cystine and methionine were the only sulphur-containing amino acids found to be present.

Conversion of methionine sulphur into cystine sulphur by Plasmodium knowlesi in vitro. When a suspension of infected erythrocytes was incubated with a chromatographically pure sample of DL-[^{35}S]methionine, this amino acid in the parasite protein became highly radioactive, while the cystine contained significant radioactivity (Table 1).

The cystine of the host-cell globin was slightly radioactive, and this may have served as a source of labelled cystine for the parasite. However, when a non-infected suspension of monkey blood (containing 6% of reticulocytes) was incubated with DL-[^{35}S]methionine in an identical manner to that described in Table 1, globin methionine contained 0·27 μC/m-mole, but no significant radioactivity was found either in globin cystine or in the intracellular free cystine and glutathione. This clearly indicates that there is no detectable conversion of methionine sulphur into cystine sulphur by erythrocytes or reticulocytes under these conditions.

It is therefore concluded that the radioactivity in cystine of the parasite protein could arise only by a conversion of methionine into cystine by the parasite. In this case, the small radioactivity in the globin cystine (Table 1) remains to be explained. In a red-cell population, only reticulocytes are known to incorporate labelled amino acids into haemoglobin (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1951), so that the diffusion of [^{35}S]cystine from parasitized cells into reticulocytes may account for this observed incorporation of [^{35}S]-cystine into haemoglobin.

Table 1. *Conversion of methionine sulphur into cystine sulphur by* P. knowlesi in vitro

A suspension of monkey blood and synthetic nutrients was incubated for 4 hr. with DL-[^{35}S]methionine (0·58 mg., 25 μC) as described in the Experimental section. This suspension contained 6% of infected red cells (Leishman stain). 10% of the red cells stained supravitally with Brilliant Cresyl Blue, i.e. parasitized red cells and reticulocytes, so that by difference 4% of the red cells are non-parasitized reticulocytes. At the end of the incubation period, cystine and methionine were isolated from the protein of the parasite and from globin as described in the Experimental section.

	Molar radioactivity of amino acid (μC/m-mole) in	
Amino acid	Parasite protein	Globin
Cystine*	0·15	0·0024
Methionine	6·49	0·049

* Calculated as μC/m-mole of cysteine.

Utilization of ^{35}S*-labelled haemoglobin by* Plasmodium knowlesi. To study the utilization of ^{35}S-labelled haemoglobin by *P. knowlesi*, labelled erythrocytes were prepared and injected into a parasitized monkey infected with large forms of the parasite which had completed about three-quarters of their cycle of development. This allowed time for complete mixing of the labelled cells with unlabelled cells already present in the monkey, before a new invasion of erythrocytes by merozoites from the ruptured mature schizonts took place. The completion of the next cycle of development of the parasites within labelled and unlabelled red cells occurred 29 hr. after the injection of the labelled cells. Re-invasion of fresh red cells again occurred. Near the end of the 24 hr. developmental period of this new generation of parasites, that is after two growth cycles had occurred in the presence of the labelled erythrocytes, the animal was bled out under anaesthesia and the parasites were isolated.

It was first necessary to determine whether the ratio of ^{35}S-labelled red cells to normal red cells remained constant over the experimental period. From Table 2 it can be seen that at first there was an increase in the number of red cells as a result of the injection of labelled cells, and then in the interval between 1·5 and 24 hr. there is an observed decrease of 8·3 %, of which not less than 5·2 % was due to rupture of the red cells by parasites. Similarly, the observed decrease of 12 % in the red-cell count in the period of 24–47 hr. can be largely attributed to destruction of cells by the parasites, in this case not less than 9·2 %. Thus there appears to be some destruction of red cells besides that attributed to the parasite, but in our opinion fluctuations in the parasite population as a result of host defences and inherent difficulties in counting red cells may make such differences of limited significance. Since the specific radioactivities of samples of the haemoglobin measured at 1·5, 24 and 47 hr. were similar, it is evident that there has not been a selective destruction of the labelled red cells. Moreover, these similar radioactivities would indicate that the production of reticulocytes has not markedly affected the proportion of labelled to unlabelled red cells. For these reasons it was concluded that the ratio of ^{35}S-labelled erythrocytes to

Table 2. *Experimental data on a monkey parasitized with* P. knowlesi *after the injection of* ^{35}S*-labelled erythrocytes*

Time after injection of labelled erythrocytes (hr.)	Erythrocytes (millions/ mm.^{3})	Parasitized erythrocytes (%)	Stages of parasite growth (% of infected erythrocytes*)				Specific radioactivity of haemoglobin (μc/g.)
			Rings	Tropho-zoites	Early schizonts	Mature schizonts	
0	5·1	7·3	0	1	20	79	—
1·5	7·2	5·2	0	0	5	95	0·018
3	—	5·5	18	0	0	82	—
6	—	8·9	93	2	0	5	0·016
24	6·6	9·2	0	0	11	89	—
29	—	26·7	98	0	0	2	—
47	5·8	23·1	0	0	23	77	0·017

* Percentage of erythrocytes infected with asexual forms of *P. knowlesi*.

Table 3. *Utilization by* P. knowlesi *of erythrocyte protein labelled with* ^{35}S *cystine and methionine*

The course of the infection in Expt. 1, in which two growth cycles of the parasite took place in ^{35}S-labelled erythrocytes, is detailed in Table 2. Other procedures are described in the Experimental section. Expt. 2 was essentially similar except that only one growth cycle of the parasite took place within the experimental period.

Amino acid or peptide	Expt. no.	Molar radioactivity of amino acid or peptide (μc/m-mole) in: Parasite protein (*A*)	Globin (*B*)	Total intracellular protein of the erythrocyte	Erythrocytes	$A/B \times 100$
Cystine*	1	0·042	0·064	0·060	—	65·6
	2	0·013	0·018	—	—	72·2
Methionine	1	0·078	0·10	0·097	—	78·0
	2	0·082	0·15	—	—	54·7
Reduced glutathione	1	—	—	—	0·013	—
	2	—	—	—	0·005	—

* Calculated as μc/m-mole of cysteine.

normal erythrocytes remained essentially constant over the experimental period.

At the end of the experimental period (47 hr.) the protein of the parasite, the haemoglobin and the total intracellular-erythrocytic protein contained significant radioactivity. The molar radioactivities of cystine and methionine isolated from these samples are shown in Table 3. It will be seen from these results that the ratio of the molar radioactivity of methionine in the parasite protein to that in globin is greater in Expt. 1 than that found in Expt. 2. At least a part of this difference may be accounted for by the following consideration. In one growth cycle, each parasite will divide into 4–16 daughter cells, with an average of 10 (Singh & Nair, 1954). Thus the final parasite material isolated would have derived more of its mass from the unlabelled parasite present at the beginning of Expt. 2, in which only one growth cycle took place, than that isolated where two consecutive growth cycles occurred.

It will be noted in Expt. 2 that the ratio of the molar radioactivity of cystine in the parasite protein to that of globin cystine is greater than that for methionine, whereas in Expt. 1 the opposite is true. The *in vitro* conversion of methionine sulphur into cystine sulphur has already been indicated, so that a similar conversion *in vivo* may account for the difference in these two experiments since the radioactivity of globin methionine was some ten times greater than that of cystine in Expt. 2, but less than twice that in Expt. 1.

Determination of sulphur compounds contained in the parasite and in an equal number of normal erythrocytes. In view of the important part played by haemoglobin in the nutrition of the parasite, it is of some consequence to determine whether the host cell contains sufficient potentially available sulphur compounds to supply the entire requirements of a parasite. For this purpose the sulphur content and sulphur-containing compounds have been estimated in a known number of mature parasites (Table 4) and the same number of normal erythrocytes (Table 5).

Table 4. *Mass and sulphur content of a known number of parasites*

Expt. 1. A monkey (2¼ kg.) with 6% of its erythrocytes parasitized was injected intraperitoneally with an aqueous solution of DL-[^{35}S]cysteine (35 mg., 20·2 μc) and the injection was repeated 24 hr. later. At 19·5 hr. after the last injection, when approximately 47% of the erythrocytes were infected with mature forms of the parasite, the animal was bled out and the parasites were isolated as described in the Experimental section. The washed, free, parasites were suspended in isotonic saline and the volume was made up to 50 ml. 48 ml. of this suspension was centrifuged at 4000 rev./min. for 10 min. in an angle centrifuge, the supernatant was removed and the residue freeze-dried. The remainder of the suspension was used to make stained smears and to determine the number of parasites/ml. Expt. 2 was a preparation of parasites from a monkey which had received no isotope.

Material	Expt. no.	Sulphur content (%)	Radioactivity (μc/mg. of sulphur)	Values for 10^{11} parasites: Mass (g.)	Values for 10^{11} parasites: Total sulphur (mg.)
Whole dried parasite material	1	0·54	—	1·01	5·45
	2	0·49	—	1·27	6·22
Parasite protein	1	0·90	0·0014	0·42	3·78
	2	0·85	—	0·54	4·59
Amino acids of the parasite protein: Cystine	1	—	0·0022	—	—
Amino acids of the parasite protein: Methionine	1	—	Nil	—	—

Table 5. *Sulphur content and sulphur-containing amino acids in the globin of monkey erythrocytes*

Monkey blood (about 25 ml.) was freed from leucocytes by centrifuging, the erythrocytes were washed 3 times in isotonic saline and the final suspension was made up to 25 ml. The number of erythrocytes/ml. was determined and the red cells in a known volume were lysed. The lysate was freed from red-cell membranes and globin isolated from the residual solution by the procedures given in the Experimental section. The protein was oxidized with performic acid, cystine and methionine being estimated in this sample by the differential-oxidation technique of Evans, 1945.

Material	Sulphur (%)	Amino acid (g./100 g. of protein)	Values per 10^{11} erythrocytes for: Total amino acid (mg.)	Total mass (g.)	Total sulphur (mg.)
Globin	0·62	—	—	2·87	17·79
Globin, oxidized with performic acid	0·59	—	—	2·70	15·93
Cystine	—	0·8*	21·5	—	5·7
Methionine	—	1·4*	38·0	—	8·2

* Mean value of three estimations.

From the results of Expt. 1 in Table 4, it is possible to calculate the cystine and methionine content of the parasite protein. This method of calculation is valid for this particular experiment since it has been shown that cystine and methionine are the only sulphur-containing compounds in the protein and that cystine sulphur is not converted into methionine sulphur when DL-[^{35}S]cysteine is administered to an infected monkey. The specific radioactivity of the parasite protein was 0·0014 μc/mg. of sulphur, which was entirely due to its cystine content. Since the specific activity of cystine isolated from this protein was 0·0022 μc/mg. of sulphur, then 1 mg. of protein sulphur must contain 0·64 mg. of cystine sulphur of this radioactivity, and, by difference, 0·36 mg. of non-isotopic methionine sulphur. From the sulphur content of the protein (0·9 %) it may be calculated that 100 mg. of parasite protein contains 2·18 mg. of cystine and 1·67 mg. of methionine.

Of the total sulphur content of the parasite, some 70 % appears to be associated with the sulphur-containing amino acids of the protein (Table 4). The non-protein residue (substances soluble in hot 5 % (w/v) trichloroacetic acid) contained inorganic sulphate, but attempts to identify organic sulphur compounds by paper chromatography were unsuccessful.

The total amount of intracellular protein contained in a known number of normal erythrocytes was determined in various ways. Thus, protein precipitation from a membrane-free lysate of red cells by 10 % (w/v) trichloroacetic acid, by heat coagulation at pH 4·5, or by an acetone precipitation, all gave similar results with a mean mass of 3·1 g. of protein/10^{11} cells. A slightly lower value (2·87 g.) was obtained for total intracellular globin by the relatively more specific method of Anson & Mirsky (1929–30). The amounts of cystine and methionine in globin oxidized by performic acid (Table 5) are similar to those obtained for globins of other mammalian species (Beach, Bernstein, Hummel, Williams & Macy, 1939). However, it is to be noted that the values given in Table 5 account for only about 85 % of the total sulphur in the protein. Indeed, by the method used, only some 83–89 % of the total sulphur could be similarly accounted for in various samples of haemoglobin and globin, a fact which may be associated with the difficulty involved in the complete oxidation of methionine (Callan & Toennies, 1941). The results shown in Table 6, in which values for recognized sulphur components of the parasite and the erythrocyte are compared, indicate that there is a large excess of cystine, methionine and glutathione in the red cell which is potentially available to the parasite.

A comparison of the uptake of ^{35}S-labelled cysteine and methionine into parasitized erythrocytes in vitro. Since the parasite *in vivo* is capable of utilizing free cysteine of the plasma (Table 4), a comparison was made of the uptake of ^{35}S-labelled cysteine and methionine from the medium by parasitized cells *in vitro* (Fig. 1). The parasite protein contains approximately twice as much cysteine as methionine (Table 6), so that the low incorporation of cysteine relative to that of methionine was not to be expected if both amino acids were freely permeable to the erythrocyte. One possible explanation is that the concentration of cysteine decreases over the experimental period due to its oxidation to cystine. The latter compound might not be permeable to the red cell or readily utilized by the parasite. However, the amount of free thiol compound remaining in the medium after 3 hr. incubation, as judged by the method of Hardin *et al.* (1954), was 73 % of that found in the beginning of the experiment. In the absence of added cysteine, infected erythrocytes produce relatively little free thiol compounds in the medium after 3 hr. incubation.

Glutathione content of parasitized erythrocytes. In five samples of monkey blood it has been estimated that there is a mean value of 9·2 mg. of reduced glutathione in 10^{11} monkey erythrocytes (cf. Table 6), a value which is in reasonable agreement with that obtained for human erythrocytes by Hardin *et al.* (1954). Estimations were then made of the glutathione content of erythrocytes parasitized with *P. knowlesi*. For example, during the development *in vivo* of young ring forms to the mature schizont stage the glutathione content of

Table 6. *A comparison of the sulphur content of equal numbers of mature forms of* P. knowlesi *and normal monkey erythrocytes*

For details of the results in this table, see Tables 4 and 5. The determination of glutathionine is described in the Experimental section.

	Sulphur content (mg.) of 10^{11} cells			
	In protein			
	Total	Cystine	Methionine	In glutathione
P. knowlesi free from host erythrocyte	3·78	2·43	1·36	—
Uninfected erythrocytes	15·93	5·7	8·2	1·01
Erythrocytes/parasites	4·21	2·3	6·0	—

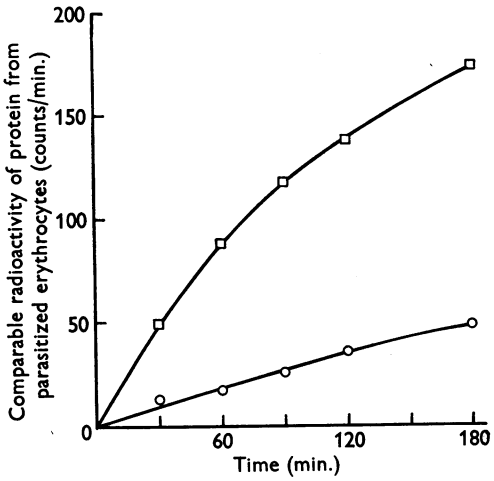

Fig. 1. A comparison of the uptake of ^{35}S-labelled cysteine and methionine by parasitized erythrocytes *in vitro*. Identical suspensions of erythrocytes (73% infected with *P. knowlesi*) were incubated for various times with equimolar amounts of either DL-[^{35}S]cysteine (0·22 μc/m-mole) or DL-[^{35}S]methionine (0·72 μc/m-mole) as described in the Experimental section. The observed radioactivities (counts/min. above background for 'infinitely thick' samples of protein, from parasitized erythrocytes, of 1 sq.cm. area) were made comparable by multiplying those of the cysteine experiment by a correction factor. This was obtained by dividing the molar radioactivity of the added methionine by that of the cysteine. □, Methionine experiment; ○, cysteine experiment.

the red cells (14% infected with *P. knowlesi*) decreased from 8·60 to 7·60 mg. of glutathione per 10^{11} erythrocytes, a fall of approximately 11·6%. In a heavier infection (22% of red cells parasitized) the content of the erythrocytes decreased from 8·90 to 7·17 mg. of glutathione/10^{11} cells, a fall of 19·4%.

DISCUSSION

Although free cystine and methionine in the medium or plasma can be used by erythrocytic forms of *P. knowlesi* (Tables 1 and 4, Fig. 1), it has also been established that the parasite utilizes sulphur-containing compounds of the red cell for the major part of its requirements for these amino acids. Thus, after the development of *P. knowlesi* through two growth cycles in ^{35}S-labelled erythrocytes *in vivo*, the cystine and methionine of the parasite protein contained 65·6 and 78·0% respectively of the molar radioactivities of these amino acids found in the globin moiety of haemoglobin (Expt. 1, Table 3). It appears, therefore, that the parasite can hydrolyse globin and utilize the degradation products for the synthesis of its own protein. This would seem likely since it has been shown that extracts of the malaria parasite of the chicken, *Plasmodium gallinaceum*, can hydrolyse haemoglobin slowly and denatured globin more rapidly (Moulder & Evans, 1946), although an attempt to demonstrate a similar proteolytic activity in extracts of *P. knowlesi* was unsuccessful (Ball *et al.* 1948).

Labelled methionine for the synthesis of parasite protein in the experiment mentioned above could only be derived from the protein of the erythrocyte, and so nearly 80% of the parasite's requirements for the amino acid is derived from this source, the remainder presumably being obtained from non-isotopic free amino acid in the plasma. This conclusion is in agreement with the observation that the growth of *P. knowlesi* within the erythrocyte *in vitro* is dependent on a supply of free methionine in the medium (McKee *et al.* 1947). Since globin in an erythrocyte contains 6·0 times as much methionine as the protein of a mature parasite (Table 6), the requirement for an extra-erythrocytic supply of this amino acid indicates that most of the globin methionine must be unavailable to the parasite. This may be occasioned by the rate of globin hydrolysis being insufficient to meet the needs of the growing parasite.

The extent to which cystine in the parasite protein is derived from any one source is, however, difficult to determine. In the *in vivo* experiment, cystine of widely differing radioactivities (Table 3) may be obtained by the parasite from protein or glutathione of the erythrocyte and by a conversion of methionine sulphur into cystine sulphur. In addition, non-isotopic cystine may be available to the parasite from the free amino acid in the plasma. For these reasons, the radioactivity observed in the cystine of the parasite protein in these experiments shows only the net result, so that the extent to which each source contributes to the cystine requirement of the parasite must be determined by other means.

Cystine of the parasite protein contains 65·6% of the molar radioactivity of globin cystine (Expt. 1, Table 3), so that it is reasonable to assume that globin can serve as a source of this amino acid for *P. knowlesi*. For the reasons given above the radioactivity measurements do not, however, give any idea of the importance of this source to the parasite, but an approximation can be made in the following manner. From the results in Table 6 it may be calculated that, for each molecule of haemoglobin hydrolysed, there will be available to the parasite methionine and cystine sulphur in the ratio 1:0·70. It is assumed that the rate of release of methionine from the hydrolysis of haemoglobin is only sufficient to provide 80% of the parasite's requirements for this amino acid, and that all methionine and cystine liberated is available and is utilized by the parasite. Thus, the ratio of methionine to

cystine sulphur released from the globin would be in the ratio $80:(80\times0{\cdot}70)=80:56{\cdot}0$, whereas the ratio required by the parasite for the synthesis of its own protein (see Table 6) would be

$$100:(100\times1{\cdot}79)=100:179.$$

On this basis 56·0/179 (= 31·3 %) of the cystine in the parasite protein could be derived from globin cystine.

The percentage decrease in the amount of reduced glutathione found in a suspension of erythrocytes is correlated with the percentage of erythrocytes infected with mature forms of *P. knowlesi* (see Results section). It is therefore possible that most of the glutathione present in an infected erythrocyte has been utilized during the growth of the parasite within the host cell. The amount of cystine sulphur contained in the glutathione of an erythrocyte has been shown to be about half that found in the protein of a mature parasite (Table 6), so that some 50 % of the cystine requirement of the parasite could be derived from this source.

Of the amino acids, only methionine is required to be present in a serum-containing medium for the growth *in vitro* of *P. knowlesi* (McKee *et al.* 1947), and this would imply that essentially all the parasite's requirements for the other amino acids are obtained from the intracellular contents of the erythrocyte. In the present experiments, [^{35}S]-cysteine injected into an infected monkey was found to be incorporated into the protein of the parasite (Table 4), and for this reason the uptake of ^{35}S-labelled cysteine and methionine into parasitized erythrocytes was compared *in vitro* (Fig. 1). However, there was a surprisingly low uptake of cysteine compared with that of methionine. This fact, together with the estimate made *in vivo* (Table 3) that only about 20 % of methionine in the parasite protein is obtained from free amino acid in the plasma, would indicate that free cysteine in the plasma or medium is a minor source of cystine to the parasite. It may be that the small utilization of plasma cysteine by the parasite (Fig. 1) is occasioned by a restricted penetration of this amino acid into the host erythrocyte. Indeed, there is some evidence (Johnson & Bergeim, 1951) which suggests that while methionine may be freely permeable to human erythrocytes, cystine and presumably cysteine are not.

P. knowlesi under *in vitro* conditions is capable of converting methionine sulphur into cystine sulphur (Table 1). It seems likely, as was indicated in the Results section (Table 3), that this conversion would take place *in vivo*, although the importance of the reaction to the parasite is not known. However, it has been indicated above that some 50 % of the parasite's cystine may be derived from glutathione of the erythrocyte, 30 % from globin cystine and a negligible amount from free cystine in the plasma, so that by difference some 20 % may be obtained by the conversion by the parasite of methionine into cystine. While such estimates can only be regarded as rough approximations, they are in general agreement with the results in Table 3, where in Expt. 1 the parasite underwent two developmental cycles within erythrocytes which contained ^{35}S-labelled globin and glutathione. While the observed radioactivity of cysteine in the parasite protein at the end of this experiment was 0·042 μc/m-mole, it may be calculated that a cysteine sample obtained from the radioactive globin cystine, glutathione and methionine of the parasite in the amounts estimated above would have a radioactivity of 0·041 μc/m-mole. Not quite such good agreement is found between the observed radioactivity (0·013 μc/m-mole) and that calculated (0·024 μc/m-mole) for cysteine in the parasite protein in Expt. 2, Table 3. In this instance, however, only one growth cycle of the parasite took place within ^{35}S-labelled erythrocytes. Thus, the observed radioactivity may be low when compared with that calculated, because the final parasite material would have derived some cystine from that present in the unlabelled parasite at the beginning of the experiment. In one growth cycle, each parasite divides into 4–16 daughter cells (Singh & Nair, 1954), so that the calculated radioactivity (0·024 μc/m-mole) should be reduced to a value 6–25 % lower than that given above (0·018–0·022 μc/m-mole) in order to take this consideration into account. Nevertheless, a difference between the observed and calculated radioactivity of cystine in the protein of the parasite still remains, so that the estimates on which the calculated radioactivity is based can only approximately fit the experimental facts in this particular experiment. The most likely explanation is that the amount of methionine converted into cystine is lower than has been estimated, since cystine available to the parasite from this source contains about 5 times the molar radioactivity of globin cystine and about 16 times that of glutathione (Table 3).

It is concluded that the essential nature of methionine for the growth of *P. knowlesi* is not only occasioned by a requirement for the preformed amino acid, but also as an additional source of cystine which the parasite cannot apparently obtain in sufficient amount from either bound cystine in the erythrocyte or the free amino acid in the plasma.

SUMMARY

1. During the growth of the malaria parasite *Plasmodium knowlesi* in ^{35}S-labelled erythrocytes *in vivo*, it has been shown that about 80 % of the

methionine in its protein is derived from that in the red-cell globin.

2. The conversion by *P. knowlesi* of [^{35}S]-methionine into [^{35}S]cystine has been shown to occur *in vitro*, and this provides a source of cystine sulphur for the parasite in addition to that already present in the haemoglobin and glutathione of the erythrocyte. Some estimates of the extent to which these sources contribute to the cystine requirement of the parasite have been made.

3. Under identical *in vitro* conditions it was found that the uptake of DL-[^{35}S]methionine by parasitized erythrocytes was markedly greater than that of DL-[^{35}S]cysteine.

4. The amounts of methionine and cystine present in the haemoglobin of a monkey erythrocyte are respectively 6·0 and 2·4 times that found in the protein of a mature parasite. The implications of this observation to the nutrition of the parasite have been discussed.

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Products of Hydrolysis of Dinitrophenylproline and *N*-Terminal Proline-peptides with Hydrochloric Acid

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During the examination of clupeine for the *N*-terminal amino acid residue (Scanes & Tozer, 1956), considerable difficulty was experienced in isolating ether-soluble dinitrophenyl (DNP) amino acid from the hydrochloric acid hydrolysate in the amount expected. A similar result was obtained by the alternative hydrolytic methods of Hanes, Hird & Isherwood (1952). As proline was suspected as an *N*-terminal amino acid, the substitution of 12N for 6N hydrochloric acid, as recommended by Porter & Sanger (1948) for use with DNP-prolyl proteins, was tried, but without success. Porter & Sanger recovered 50% of the DNP-proline added to globin before hydrolysis, but other workers have reported much lower recoveries, namely, 20% from a collagen hydrolysate