THE UPTAKE OF RADIOACTIVE PHOSPHORUS BY ERYTHROCYTIC STAGES OF *PLASMODIUM BERGHEI* (VINCKE & LIPS)

By P. R. WHITFELD*

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Summary

Radioactive phosphorus (^{32}P) was injected intraperitoneally into mice infected with *Plasmodium berghei* and the activity of the phosphorus-containing fractions of the parasites was determined at intervals during the subsequent 48 hr.

The lipid fraction had the highest content of ^{32}P; it contained half of the total ^{32}P present in the parasites. The ^{32}P content of desoxyribonucleic acid (DNA) was 3.5 per cent. of the total ^{32}P at the end of 2 hr, but increased to 24 per cent. of the total at the end of 48 hr. After 2 hr, the acid-soluble ^{32}P accounted for 23 per cent. of the total ^{32}P, but after 48 hr it accounted for only 13 per cent. At all times, 10 per cent. of the total ^{32}P was contained in the ribonucleic acid (RNA). The ^{32}P content of the phosphoprotein fraction was very low throughout the experiment.

The total ^{32}P content of the parasites after 48 hr was approximately four times the content after 2 hr.

I. INTRODUCTION

Most observations on the chemical properties of the erythrocytic stages of malaria parasites have been comparative in nature. The properties of the host's normal red blood cells have been compared with the properties of the infected red blood cells and any differences between them have been attributed to the parasites. Although much has been learnt by this method (Ball 1946; Ball *et al.* 1948), the results only indicate the apparent properties of the malaria parasite. No allowance can be made for the associated change in the properties of the red cells caused by the presence of the parasites.

Moreover, certain types of experiment by this technique are impracticable. For instance, in an experiment designed to show the incorporation of radioactive phosphorus into a malaria parasite, the activity of the host red blood cell would be so great, compared with that of the parasite, that the accuracy of the results must suffer.

The development of a quantitative procedure for the isolation of malaria parasites, uncontaminated with host material, has made possible the direct study of the chemical properties of these parasites. This paper describes the application of this technique to the examination of the uptake *in vivo* of radioactive phosphorus by *Plasmodium berghei*.

* Division of Animal Health and Production, C.S.I.R.O., McMaster Laboratory, Sydney.

II. Materials and Methods

The strain of *P. berghei* used in these experiments has been described previously (Whitfeld 1953). Blood was collected from infected mice by anaesthetizing them lightly and then severing the jugular vein. Powdered heparin was used as an anticoagulant.

Red blood-cell counts were carried out on a Spencer Bright Line hemacytometer and parasite counts were made on thin blood smears stained with Leishman's stain; enough cells were counted to reduce the error to less than 10 per cent. (Whitfeld 1953).

A modification of the procedure of Davidson, Frazer, and Hutchinson (1951) was followed for the separation of the various phosphorus-containing fractions of the parasites.

The agglutination of the red blood cells during the procedure for the isolation of the parasites was effected with "Intradex" (6.0 per cent. depolymerized "Dextran" in 0.9 per cent. sodium chloride) which was kindly supplied by The Crookes Laboratories, London.

Radioactive phosphorus was used as sodium dihydrogen phosphate in phosphate-saline (0.85 per cent. saline, 0.05M with respect to phosphate, pH 7.3). Radioactivity determinations were carried out on 16.0-ml samples in a liquid counter (Type M 12, 20th Century Electronics) attached to a scale of eight. Three 5-min counts, alternating with two 5-min background counts, were taken; this was sufficient to reduce the error to 5 per cent. in the lowest counts recorded, and to correspondingly less in higher counts. Corrections for ^{32}P decay were made when necessary. A standard solution of potassium carbonate (100 g per 100 ml) was used for testing variations in the sensitivity of the counter.

III. Procedure and Results

For each experiment 100 white mice were infected with *P. berghei* and blood films were made daily and examined. When approximately 20 per cent. of the red blood cells were parasitized, 4μc ^{32}P per 25 g body weight were injected intraperitoneally into each mouse.

Five minutes after the ^{32}P injection the blood was collected from three mice and centrifuged. A 0.5-ml sample of the plasma was taken and 1.0 ml ice-cold 10 per cent. trichloracetic acid was added. After 30 min at 0°C the precipitated protein was spun down and the supernatant set aside for the determination of the initial level of inorganic ^{32}P in the host plasma.

The remaining mice were divided into seven groups, each of which contained 12-14 mice. One group was killed after 2 hr, another after 4 hr, the others after 6, 8, 12, 24, and 48 hr, and for each group the pooled blood was centrifuged and the plasma removed. Of the plasma, 0.5 ml was kept for the determination of plasma inorganic ^{32}P. Two volumes of ice-cold phosphate-saline and 0.6 volumes of "Dextran" were added to the packed red cells, which were then resuspended and placed in the refrigerator. When the red cells had settled (20 min or less) the supernatant, which contained a few red cells and the majority of the leucocytes, was discarded. After resuspension in phosphate-saline the red cells were spun down lightly and the supernatant was

discarded. A thin smear of the red cells was stained and examined immediately for leucocytes, and subsequently for the determination of the parasite count. If the number of leucocytes exceeded 0.01 per cent. of the red cells the cells were resuspended and the treatment with "Dextran" was repeated. Finally they were resuspended in saline to give a total volume of 10.0 ml and a red blood-cell count was carried out.

Of this supension, 2.0 ml was taken for total ^{32}P determination and the other 8.0 ml for separation into phosphorus-containing fractions. Both samples were hemolysed with saponin (Bovarnick, Lindsay, and Hellerman 1946) and the parasites freed from the ghost red blood cells by careful centrifugation. The parasites were washed with ice-cold saline till the washings showed no activity.

TABLE 1

ACTIVITY OF PLASMA INORGANIC PHOSPHORUS OF MICE INFECTED WITH *P. BERGHEI* AFTER INTRAPERITONEAL INJECTION OF 4μC ^{32}P/25 G BODY WEIGHT

Hours after ^{32}P Injection	0·1	2·0	4·0	6·0	8·0	12·0	24·0	48·0
Activity (counts/min/ml plasma)	30900	7720	3885	2340	2080	1860	1230	840

(*a*) ^{32}P *Content of Mouse Plasma Inorganic Phosphate*

A suitable aliquot of the trichloracetic acid extract of the plasma was treated with acid molybdate in the presence of sodium chloride and extracted with butanol (Ennor and Stocken 1950). The activity of the butanol fraction was measured. The results are given in Table 1.

(*b*) *Total* ^{32}P *Content of* P. berghei

The parasites were ashed with a mixture of nitric, perchloric, and sulphuric acids (8:1:1 by volume). After neutralization the volume was made up to 16.0 ml and the activity determined (Table 2).

(*c*) ^{32}P *Content of Phosphorus Fractions of* P. berghei

The parasites were extracted three times with ice-cold 10 per cent. trichloracetic acid; the extracts were combined and made up to a definite volume and the activity was measured. In the first experiment a fourth acid extraction was carried out and its activity determined. However, as this was found to be negligible, only three extractions were made in later experiments.

The residue was then extracted successively with acetone, alcohol, chloroform, and alcohol-ether (3:1) (twice) to remove the lipid fraction. The activity of the second alcohol-ether extract was determined separately and in every test was insignificant. After evaporation to dryness the lipid fraction was re-extracted with chloroform and the activity measured.

DNA (1.0 mg) was added to the acid-extracted, lipid-free residue, which was then digested overnight at 37°C in 1N KOH. The DNA was precipitated with acid-methanol and purified by redissolving in alkali and reprecipitating three times before it was finally dissolved and made up to 16.0 ml for determination of the ^{32}P content.

The supernatant, after precipitation of DNA, contained ribonucleotides and inorganic phosphorus which was derived from phosphoprotein. These two fractions were separated by butanol extraction in the presence of acid molybdate and sodium chloride. The activity of the butanol layer was then a measure of phosphoprotein ^{32}P and the activity of the aqueous layer was a measure of RNA ^{32}P.

TABLE 2

UPTAKE OF ^{32}P BY PHOSPHORUS FRACTIONS OF *P. BERGHEI**

Fraction	Hours After Injection of ^{32}P into Host						
	2·0	4·0	6·0	8·0	12·0	24·0	48·0
Acid-soluble P	100	115	207	220	186	149	246
Lipid P	244	466	571	482	558	574	995
RNAP	45	68	56	70	98	115	187
DNAP	15	82	62	190	191	322	457
Phosphoprotein P	30	18	28	24	21	13	12
Total P (sum of fractions)	434	748	924	986	1054	1173	1897
Total P (experimental)	482	779	1009	1120	1196	1165	2086

* Activities are expressed as counts/min/10^{10} parasites.

Total ^{32}P content and ^{32}P content of the different fractions of *P. berghei* are given in Table 2. Results are expressed as counts per minute per 10^{10} parasites; each result is the average of at least two, but usually four, experiments.

It will be noted that at all times except in the 24-hr experiment the total ^{32}P content, calculated as the sum of the fractions, is less than that determined experimentally. This discrepancy, however, is not great and no doubt is due to small losses incurred during the long fractionation procedure.

The possibility of contaminating activity due to leucocytes in the parasite preparation was excluded by the following experiment. Five normal mice were each injected with 4 μc ^{32}P and their blood was collected 24 hr later. After removing the plasma, the cells were resuspended in phosphate-saline and "Dextran" was added. When the red cells had separated out the supernatant was discarded and the cells were washed once with saline and hemolysed. The ghost red blood cells were removed by careful centrifugation and the microscopical residue was washed with saline. The total ^{32}P content of this residue was negligible. It was concluded that the results given in Table 2 are a true indication of the ^{32}P content of the parasites.

An average dose of 4 μc ^{32}P per mouse might be considered larger than desirable but preliminary experiments had indicated that, unless a dose of this size was used, the activity of some of the fractions in the earlier stages was too low for measurement. An examination of stained preparations from dosed and non-dosed mice showed that neither parasite nor red blood-cell morphology was affected by the radioactivity.

IV. Discussion

It should be emphasized that the results given above are not expressed as specific activities. Thus, although the lipid fraction has the highest ^{32}P content, it does not necessarily follow that it has the highest specific activity. Similarly the ^{32}P content of the phosphoprotein is very low but its specific activity is probably quite high.

An examination of the variations with time in the relative amounts of ^{32}P in the different parasite fractions yields more information than an examination of the individual absolute ^{32}P contents at any particular time. These variations are emphasized if the ^{32}P content of the parasite fractions is expressed as a ratio of the host plasma inorganic ^{32}P (Table 3).

Table 3

RELATION BETWEEN ACTIVITY OF PHOSPHORUS FRACTIONS OF *P. BERGHEI* AND ACTIVITY OF HOST PLASMA INORGANIC PHOSPHORUS*

Fraction	Hours After Injection of ^{32}P into Host						
	2·0	4·0	6·0	8·0	12·0	24·0	48·0
Acid-soluble P	1·3	3·0	8·8	10·6	10·0	12·1	29·2
Lipid P	3·2	12·0	24·4	23·2	30·0	46·6	118·0
RNAP	0·6	1·7	2·4	3·3	5·3	9·3	22·2
DNAP	0·2	2·1	2·7	9·1	10·3	26·1	54·0
Phosphoprotein P	0·4	0·5	1·2	1·2	1·1	1·1	1·4
Total P (sum of fractions)	5·7	19·3	39·5	47·4	56·7	95·2	224·8

*Values are expressed as the ratio: $\frac{\text{activity of parasite fraction (counts/min/}10^{10}\text{ parasites)}}{\text{activity of host plasma inorganic P (counts/min/ml plasma)}} \times 100.$

Several interesting points become apparent. The lipid fraction has the highest ^{32}P content; it contributes approximately 50 per cent. of total ^{32}P and its value after 48 hr is 36 times its 2-hr value. On the other hand, the fraction in which the greatest increase in ^{32}P content occurs between 2 and 48 hr is the DNA; the 48-hr value of DNA ^{32}P is 270 times the 2-hr value and the contribution to total ^{32}P rises from 3.5 per cent. after 2 hr to 24 per cent. after 48 hr. As the ^{32}P content of RNA remains at about 10 per cent. of total ^{32}P during the 48-hr period, the ratio of RNA ^{32}P to DNA ^{32}P changes. Thus, after 2 hr the ratio is 3.0, but from 4 to 6 hr the ratio is 1.1 and after 8 hr it falls to 0.4.

The rate of incorporation of ^{32}P is faster during the early stages of the experiment. Between 24 and 48 hr the value of each fraction, except phosphoprotein, increases about 2.5-fold, whereas between 2 and 6 hr the values increase from fourfold (RNA) to 13-fold (DNA). Moreover, there seems to occur what might be termed a "lag-phase." From Table 3 it can be seen that there is very little increase in the values for the acid-soluble and DNA fractions during the period 8-12 hr, and for the lipid fraction during the period 6-8 hr. As schizogeny in *P. berghei* occurs between 6.00 a.m. and 10.00 a.m. (Ramakrishnan and Prakash 1951) and the experiments were always started about 9.00 a.m., then the period 6-12 hr later would correspond to the late trophozoite stage. Apart from this, there seems to be little point in attempting to correlate the changes in ^{32}P content of the fractions with some definite stage in the parasite's growth cycle.

Since this work was completed a paper has been published on the incorporation of ^{32}P into *Plasmodium gallinaceum* (Clarke 1952). In this paper the values are derived from differences between parasitized and non-parasitized red blood cells, but a comparison with the values presented above shows reasonable agreement between the two sets of results.

The possibility of a relationship between the mode of action of certain anti-malarials and parasite nucleic acids has been suggested by Parker and Irvin (1952), who demonstrated the binding power of chloroquine on DNA and RNA. An examination of the ^{32}P uptake by the nucleic acids of malaria parasites treated with chloroquine might shed some light on this problem. Furthermore, a comparison of the ^{32}P content in the nucleotides of nucleic acids isolated from normal and resistant strains of parasites is now possible and might lead to some interesting results.

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