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An Aspect of the Ribonucleic Acid Synthesis *in Vitro* in Free Parasites of Malaria (*Plasmodium berghei*)

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An aspect of ribonucleic acid (RNA) synthesis in malaria (*Plasmodium berghei*) was observed using free parasite cells *in vitro*. The time course of the incorporation of uridine-2-^{14}C into parasite RNA showed a linearity up to one hour and then a plateau. The incorporation rate was directly proportional to the amount of malarial parasites used. The rate and the level of the uridine incorporation into parasite RNA were higher in K^+-rich medium than in Na^+-rich medium. Coenzyme A and dithiothreitol had no stimulative effect on the uridine incorporation into parasite RNA. Their addition had no protective effect also on the preservation of the RNA synthesizing capability of free parasites. The addition of adenosine, guanosine, and cytidine to the incubation mixture inhibited apparently the incorporation of uridine-2-^{14}C into parasite RNA. When cytidine was omitted from those additions, no inhibition was observed. Dinitrophenol inhibited only 30% of the uridine incorporation and no further inhibition was observed by the further addition of the inhibitor. Actinomycin D inhibited 80% of the uridine incorporation at 0.5 μg/ml and that shows, in *Plasmodium berghei*, RNA synthesis is desoxyribonucleic acid (DNA)dependent.

Malaria is an obligate intracellular parasite. The reason why malaria is an obligate one is not known. The others that belong to a group which requires unknown factors satisfied only within living cells are listed only viruses, rickettsiae, or so. In this meaning, malaria is very unique one, because it belongs to protozoa (much more evolved than viruses), but it can live only within living cells.

Many works have been done on the molecular biology of bacteria and vertebrates in detail, but very few are known on the molecular biology of protozoa, much less of malaria.

The studies on the effect of drugs on the protein synthesis in malaria were undertaken, and a series of them — amino acid activation for protein synthesis in malaria,[2] biogenesis of ribosomes in malaria,[3] phosphorylation of D-arabinosyl adenine by malaria and its protective effect on the infection,[4] and so on—had been done. As one of them, an aspect of the ribonucleic acid (RNA) synthesis in malaria was characterized. It is hoped that an understanding of the uniqueness of the biology of this organism will help to control malaria.

Experimental

Maintenance of the Infection——The NYU-2 strain of the rodent malaria, *Plasmodium berghei*, was maintained in young female mice weighing 20—22 g by the passage of the infected blood twice a week as described by Wellde, *et al.*[5] The concentration of infected blood was adjusted by diluting the blood usually 1 to 5 with ice-cold saline-citrate buffer (127 mM NaCl, 35 mM citrate buffer), so that on the 4th day after inoculation the parasitemia was usually 75—80%. Parasitemia was determined by counting Giemsa stained smears.

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2) J. Ilan, Judith Ilan, and K. Tokuyasu, *Military Med.*, **134**, 1026 (1969); Judith Ilan and J. Ilan, *Science*, **164**, 560 (1969).
3) K. Tokuyasu, J. Ilan, and Judith Ilan, *Military Med.*, **134**, 1032 (1969).
4) J. Ilan, K. Tokuyasu, and Judith Ilan, *Nature*, **228**, 1300 (1970).
5) B.T. Wellde, N.T. Briggs, and E.H. Sadun, *Military Med.*, **131**, 859 (1966).

Blood Collection and Handling——The infected blood was collected by heart puncturing into a sterile syringe containing 1 ml of ice-cold saline-citrate buffer and immediately mixed with 10 volumes of the same medium. The citrate-treated blood, usually 14—15 ml of infected blood by bleeding 20 infected mice, was centrifuged at $800 \times g$ for 5 minutes. The plasma and the white blood cells, settled as buffy coat above the red blood cells, were removed by means of a Pasteur pipet. This cleaning procedure was repeated 4—5 times in the cold room.

Preparation of Free Parasites——The infected red blood cells separated as mentioned above were then treated with enough volumes of ice-cold hypotonic medium (0.4% NaCl or 0.5% KCl) and centrifuged at $1500 \times g$ for 5 minutes. The brown packed cells of free parasites were washed 4 times with the same medium and the white ghosts, settled above the free parasites, were removed by means of a Pasteur pipet before each resuspension. Microscopic observations showed no red blood cells and almost no ghosts after washing. The contamination of the white blood cells in the free parasites was denied by the careful microscopic observations after Giemsa staining. The free parasites obtained were able to infect mice. The yield of free parasites was 0.2 ml of packed cells from 10 mice.

Incubation *in Vitro*——The infected red blood cells were suspended to 4 volumes of isotonic modified Krebs–Ringer solution (123 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 16 mM Na_2HPO_4, pH 7.4) containing 10 mM glucose. The free parasites were suspended to the same medium (usually 20—30 mg protein/ml). Cell suspension of 0.8—1.0 ml was maintained in a 10 ml Erlenmeyer flask and 1 μCi of uridine-2-^{14}C (purchased from New England Nuclear Corp., Boston; 26 mCi/m mole) was added to each flask. Incubations were carried out at 37° by gentle shaking under 1 atmosphere of oxygen. The incubation was terminated by the addition of 5% cold trichloroacetic acid (TCA). After standing in ice-bath for 30 minutes, the precipitate was washed 4 times with 5% cold TCA and twice with ice-cold ethanol. Washing was done in the cold room. The precipitate was suspended in ether and transferred to counting vials. After drying, 5 ml of toluene containing 0.5% 2,5-diphenyl oxazole and 0.03% 1,4-bis[2-(4-methyl 5-phenyloxazolyl)] benzene was added and the radioactivity of the precipitate was measured with a liquid scintillation counter (Model 6822, Nuclear Chicago Corp.). The protein concentration was determined colorimetrically according to Lowry, *et al.*[6] using biuret and phenol reagents.

Result and Discussion

An *in vitro* system for RNA synthesis was obtained employing the infected red blood cells. The time course of the incorporation of uridine-2-^{14}C into parasite RNA in the infected red blood cells showed a linearity up to one hour and then a plateau. Since the red blood cells of normal mouse are devoid of nuclei and RNA synthesis is generally desoxyribonucleic acid (DNA) dependent, the time course of incorporation of radioactive precursors into RNA of infected red blood cells can be regarded to indicate the rate of RNA synthesis in parasites.

The nucleic acid metabolism of malaria is thought to be similar to that of other organisms. The increases of nucleic acids in the infected red blood cells are thought to have come from the *de novo* synthesis of them in the malarial parasites from the precursors of non-erythrocytic origin. However, no critical experiments with isolated parasites have been done yet.

An *in vitro* system for RNA synthesis by free parasites was, therefore, prepared to observe directly the incorporation of radioactive precursors into free parasite RNA or to eliminate the effect of other possible factors which can influence the incorporation rate. The time course of the incorporation of uridine-2-^{14}C in free parasites is given in Fig. 1. The incorporation was nearly linear for one hour and then the rate was decreased, similar to the results obtained using the infected red blood cells. The effect of altering the amount of parasites is given in Fig. 2. It was observed that the incorporation was directly proportional to the amount of parasites used.

Na^+ and K^+ ions are known to be intimately involved in the active transport of various substances and in the regulation of the activities of certain enzymes. So, an attempt was made to observe the effect of K^+ concentration on the incorporation rate of uridine into parasite RNA. When NaCl was replaced by KCl (123 mM) in the incubation medium, a 30—40% increase in the incorporation as well as a higher initial rate of incorporation was observed (Fig. 3). While the Na^+ and K^+ concentrations are 142 and 5 meq/liter in the plasma, those of Na^+ and K^+ concentrations are known to be 1 and 108 meq/liter respectively, in the

6) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

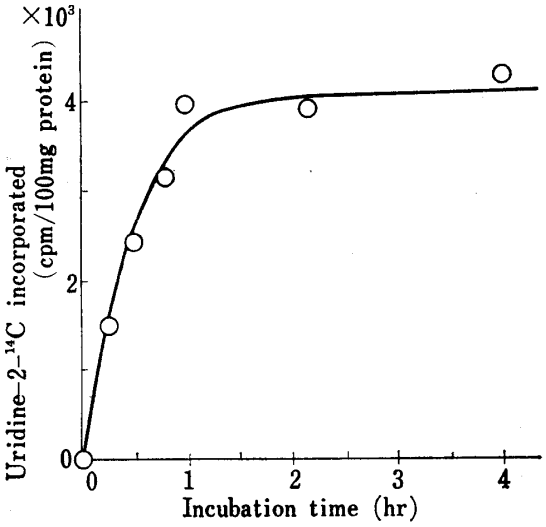

Fig. 1. Time Course of the Incorporation of Uridine-2-^{14}C into RNA of Free Parasite Cells *in Vitro*

Suspension of free parasite cells was incubated by gentle shaking at 37° for various periods of time in a modified Krebs-Ringer phosphate medium, pH 7.4, containing 10 mM glucose and 1 μCi of uridine-2-^{14}C. The total volume was 1 ml. The gas phase was O_2.

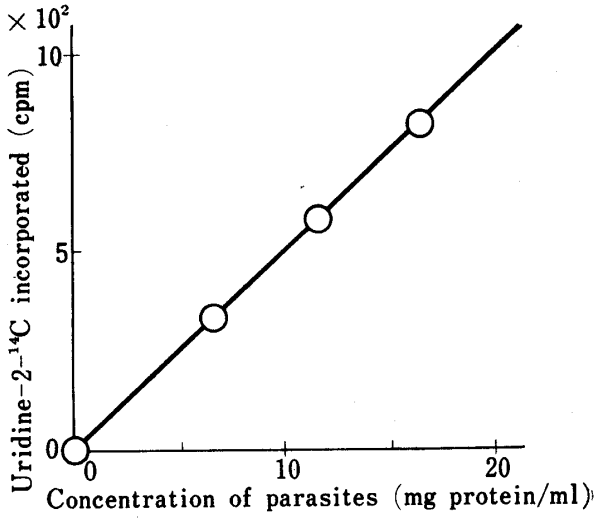

Fig. 2. Effect of Parasite Cell Concentration on the Incorporation of Uridine-2-^{14}C into Parasite RNA

Experimental conditions were as given in Fig. 1. Incubation time was 1 hr.

red blood cells. On the other hand, the Na^+ and K^+ concentrations were 155 and 5 meq/liter in the Na^+-rich medium and those of Na^+ and K^+ ions were 37 and 123 meq/liter, respectively, in the K^+-rich medium used in the present experiment. The results shown in Fig. 3. seem to indicate that the Na^+ and K^+ concentrations in the red blood cells are preferable for the malarial parasites to synthesize their RNA to those in the plasma.

According to Trager,[7] coenzyme A (Co A) is the essential factor for the survival of parasites outside the red blood cells. Dithiothreitol (DTT) is frequently used to protect enzymes (or thiol compounds) against inactivation caused by the oxidation of thiol groups or by traces of heavy metals. So, the effect of the addition of Co A with or without DTT to the suspension of free parasites was observed. However, they exhibited no stimulative effect on the uridine incorporation and no protective effect on the preservation of the RNA synthesizing capability of free parasites. The free parasites lost approximately 60% of their synthesizing activities after standing overnight at 2—4° with or without Co A and DTT.

The addition of other cold nucleosides to the incubation medium was expected to enhance the incorporation rate of radioactive uridine into parasite RNA. However, the addition of cold adenosine, guanosine, and cytidine to the medium inhibited apparently the incorporation of uridine-2-^{14}C into parasite RNA. When free parasites were incubated with radioactive uridine, and only cold adenosine and guanosine were supplemented (cold cytidine omitted), no inhibition was observed as shown in Fig. 4. These results are supposed to indicate that the malarial parasites have the capability to convert (deaminate?) cytidine to those that cause the isotopic dilution of uridine-2-^{14}C. However, no further criteria to ascertain the supposition was obtained.

By the addition of dinitrophenol to the incubation mixture, an only 30% inhibition of the uridine incorporation was observed at the concentration of 0.1 mM and no further inhibition was observed by the further addition of dinitrophenol (Fig. 5). This fact shows that the energy formation through oxidative phosphorylation is only partly required for the incorporation of uridine into parasite RNA. When actinomycin D was added to the incubation mix-

7) W. Trager, *J. Exp. Med.*, **96**, 465 (1952); W. Trager, *J. Protozool.*, **1**, 231 (1954).

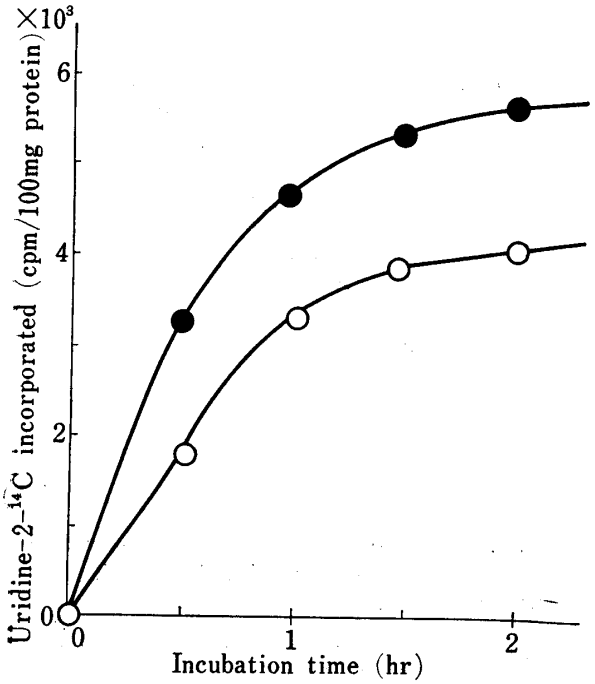

Fig. 3. Effect of K^+ Concentration on the Incorporation of Uridine-2-^{14}C into Parasite RNA

Experimental conditions were as given in Fig. 1 except Na^+ and K^+ concentrations in incubation medium. ○: Na^+-rich medium (123 mM NaCl and 5 mM KCl); ●: K^+-rich medium (5 mM NaCl and 123 mM KCl)

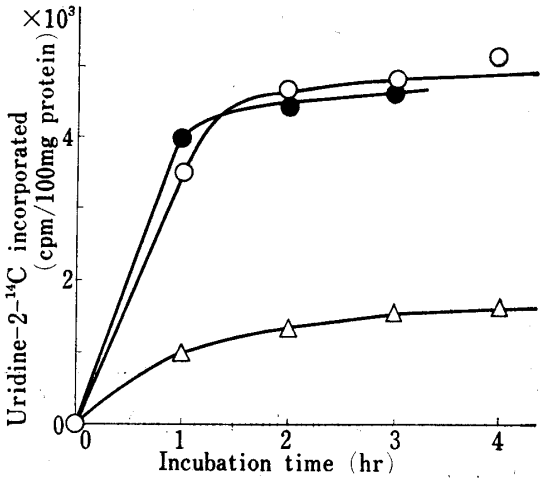

Fig. 4. Effect of Other Nucleosides on the Incorporation of Uridine-2-^{14}C into Parasite RNA

Experimental conditions were as given in Fig. 1 except the addition of other cold nucleosides into incubation medium. The final concentrations of other nucleosides were 0.1 mM, respectively. ○: no other nucleosides; ●: adenosine+guanosine; △: adenosine +guanosine+cytidine

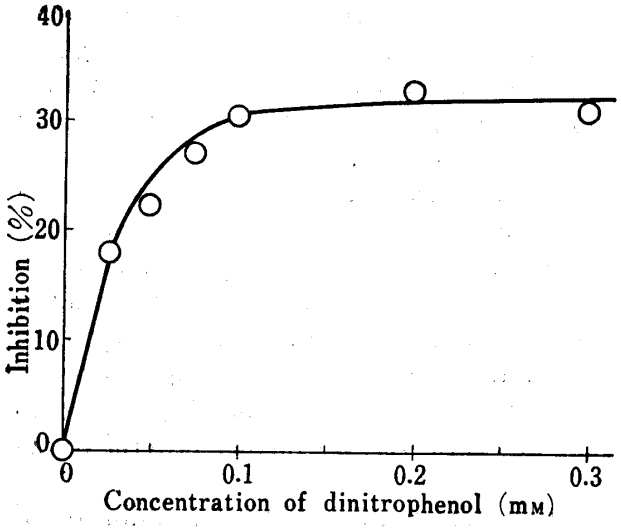

Fig. 5. Effect of Dinitrophenol on the Incorporation of Uridine-2-^{14}C into Parasite RNA

Experimental conditions were as given in Fig. 1 except the addition of dinitrophenol into incubation medium. Incubation time was 1hr.

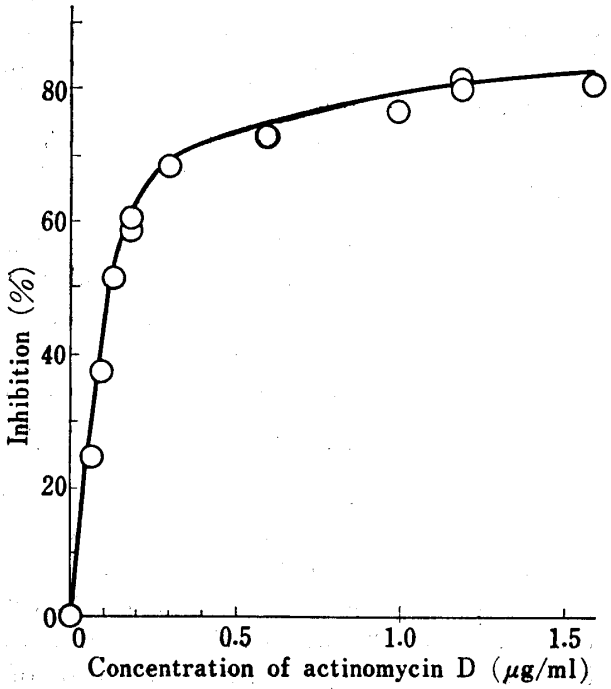

Fig. 6. Effect of Actinomycin D on the Incorporation of Uridine-2-^{14}C into Parasite RNA

Experimental conditions were as given in Fig. 1 except the addition of actinomycin D into incubation medium. Incubation time was 1 hr.

ture, there was a linear inhibition of the uridine incorporation in the range of 0—0.2 μg/ml of actinomycin D and an 80% inhibition at 0.5 μg/ml as shown in Fig. 6. These results indicate that the uridine incorporation into parasite RNA is DNA dependent.

The malarial parasite resembles viruses in that it is an obligate intracellular parasite. While no characteristic differences between the RNA synthesis of the malarial parasite and that of other organisms was shown by the present experiments, there is a suggestion that the malarial parasite synthesizes only transfer RNA, messenger RNA, and 40S ribosomal subunits and utilizes the host 60S ribosomal subunits.[3] There might be one of the reasons why malaria is an intracellular parasite.

On the other hand, there are some speculations to the reason of the restriction of the malarial parasite to an intracellular existence, *i. e.*, the defect of CoA synthesis, the defect of carbohydrate oxidation, permeability defect of the parasite, and so on. There will be needed more detailed experiments to elucidate the reason why malaria is an obligate intracellular parasite.

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