THE USE OF PHOSPHORUS 32 IN STUDIES ON PLASMODIUM GALLINACEUM

II. Studies on Conditions Affecting Parasite Growth in Intact Cells and in Lysates

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(Received for publication, June 24, 1952)

In the preceding paper (1), the development of a quantitative method for determining the extent of the growth of the erythrocytic form of *Plasmodium gallinaceum in vitro* has been described. This method utilizes phosphorus, P^{32}, to measure the extent of the incorporation of phosphorus into nucleic acids, in particular desoxypentose nucleic acids. It was found that the degree of phosphorus incorporation into the DNA fraction could be related to the extent of parasite growth and development observed in stained films. It was suggested that such a method offers the advantages of objectivity, sensitivity, and quantitation, which are not so readily obtainable from the comparative examination of stained films. In the present paper this technique has been applied to the study of the effect of various conditions on parasite growth *in vitro*.

In choosing the materials for study, we were influenced by the work of Anfinsen, Geiman, and their associates working at Harvard (2), by the work of Trager (3–6), and by the results obtained by Whitman in these laboratories (7). The Harvard group of investigators devised a synthetic medium for use in the cultivation of the simian parasite, *P. knowlesi*. Trager carried out studies on *P. lophurae* using both media containing extracts of normal avian erythrocytes and a slightly modified form of the Harvard medium. Whitman found an extract of normal chicken erythrocytes to be markedly favorable to the survival of the sporozoite form of *P. gallinaceum in vitro*. In our studies, the approach differed from that of other investigators in that we were not concerned with the problem of reinvasion, but, rather, our observations were confined to a single generation of parasites over a period of 18 to 22 hours, the parasite used having an asexual cycle time of approximately 36 hours. Our aim was to obtain a simplified system, free of large numbers of normal cells, in which the parasitized cell could be investigated under controlled conditions.

EXPERIMENTAL

Methods and Materials

Medium for Lysate Studies.—Our work was carried out with dilute suspensions (1 to 5 per cent) of moderately heavily parasitized erythrocytes which had been centrifuged from heparinized blood. The preceding paper gives the descriptions of the use of the isotope, the methods of obtaining and handling the parasitized cells, the technique of incubation, and the methods used in the chemical fractionation and radioassay. The same paper gives the basic methods used in the preparation of erythrocyte extracts from normal chicken cells. For intact cells these extracts were used at concentrations of 20 to 30 per cent. The medium which was finally developed for work with lysates consisted of a 50 per cent freshly prepared red cell extract patterned after that of Trager (9) with regard to the method of obtaining and handling the cells and the high potassium concentration of the diluent. To the freshly thawed cells an equal volume of diluent was added which had the following composition: 0.0508 M $NaCl$, 0.0265 M KCl, 0.001 M $MgSO_4$, 0.0002 M $MnCl_2$, 0.0141 M $NaHCO_3$, 0.02 M glucose, 0.02 M malate (which was equimolar with respect to sodium and potassium), and 0.005 M hexose diphosphate (magnesium salt). The resultant mixture was centrifuged and the supernatant extract secured as has been described in the preceding paper.

Preparation of Lysates of Parasitized Cells.—The preparation of lysates of parasitized cells followed the techniques of Speck *et al.* (8) and of Trager (9), employing specific rabbit anti-chicken red cell hemolysin and guinea pig complement. Sterile stocks of such sera were prepared and stored frozen in a dry ice cabinet. Once thawed, these sera were not refrozen because in our experience the activity of such materials was unreliable. Lysis was carried out as routine with parasitized cell suspensions at a concentration of 10 per cent, the minimal quantity of hemolysin and complement necessary to produce lysis of all cells being employed. These quantities were found to vary markedly with the medium employed to suspend the cells, increasing quantities being necessary as the concentration of erythrocyte extract in the medium was increased. The presence of any significant quantity of chicken serum in the lysis medium almost invariably resulted in incomplete lysis and was avoided as routine. Lysis was carried out at 40°C. in an incubator, vigorous rotation throughout the 30 minute lysis period being maintained by attachment of the flask to a rotary shaker.[1] At the end of the period of lysis, the contents of the flask were centrifuged for 10 minutes at 2,500 R.P.M., the supernatant fluid discarded, and the sediment, containing the parasites and erythrocyte stroma and nuclei, was distributed by pipette to flasks of media and P^{32}. An amount of sediment equivalent to 0.5 to 1.0 ml. of intact cells was used for each flask, the final concentration being equivalent to 5 to 10 per cent intact cells.

Special Media Components.—The synthetic medium employed was patterned after that used by Anfinsen and his associates (2). This was used without plasma, 1 per cent crystallized bovine albumin (Armour) being used as the source of protein. The latter was dissolved in water, neutralized with dilute NaOH, dialyzed against glass-redistilled water, and filtered to sterilize prior to use. The concentration of inorganic phosphate was kept low to avoid excessive dilution of the isotope and was adjusted to approximate that of the medium containing erythrocyte extract (10 to 30 mg. per cent phosphorus). The amino acids, vitamins,[2] purines, pyrimidines, glucose, glycerol, and sodium acetate were used as described by the original authors.

Where normal chicken serum was used in the medium, it was obtained by one of two methods. Serum free of red cell material (as judged by detectable hemoglobin) was secured

[1] Fisher-Kline Rotator, Fisher Scientific Company.

[2] We are indebted to Merck and Company for the cocarboxylase used in these studies.

by drawing blood without anticoagulant and running it gently into chilled tubes free of etching. After centrifugation in the cold, the supernatant plasma was removed and defibrinated by shaking with glass beads and warming to 37°C. This method was necessitated by the fact that chicken blood shows very poor clot retraction even over periods of many hours. Where contamination with erythrocyte material was unimportant, serum was obtained as a by-product in the preparation of red cell extract by the defibrination technique of Trager (9). In either case the serum was heated before use for 1 hour at 56°C., reinforced by the addition of glucose, and brought to a pH of approximately 7.2 with 100 per cent gaseous CO_2.

The various other chemical entities added to the media were obtained commercially and were recrystallized where indicated and feasible.

Anaerobic Conditions.—Anaerobic conditions were obtained by treatment of a tanked mixture of 5 per cent CO_2–95 per cent N_2 with yellow phosphorus in a sealed 5 gallon carboy for a period of 3 to 7 days. The incubation flasks were rendered air-tight by the use of paraffin seals and sealed water traps connected to both entrance and exit tubes. The air contained in the system was flushed out by a rapid flow of the treated N_2–CO_2 for a period of 30 minutes or more, following which a slow flow of the gas was maintained for the duration of the experiment.

A discussion of the method of expressing the results as micrograms of phosphorus incorporated per milligram of nucleic acid phosphorus is to be found in the preceding paper.

RESULTS

Studies with Intact Cells

Comparison of Synthetic Medium and Erythrocyte Extract.—The first results to be reported compare the effect of the synthetic medium designed by Anfinsen and his associates with a medium consisting of 20 per cent erythrocyte extract to which had been added all the various vitamins, amino acids, purines, and pyrimidines used for the synthetic medium. The cells were incubated as 1 per cent suspensions for 18 to 20 hours. In Table I the results of two experiments are presented, one giving the total nucleic acid values and the second showing the results when the nucleic acids are separated into pentose nucleic acids (PNA) and desoxypentose nucleic acids (DNA).

Although some growth was obtained with the synthetic medium, it is obvious that the medium containing erythrocyte extract was markedly superior. The extent of this superiority is better revealed in Experiment B, in which an almost sixfold increase in the DNA fraction is observed. Although the activity of the total nucleic acid fraction provides a reasonable comparative index of parasite growth in intact cell systems, the activity of the DNA fraction, as has been discussed in the preceding paper, appears to be a more specific and sensitive method of measurement. The poor results obtained with the synthetic medium relative to those with the cell extract indicated that the latter possessed an activity not attributable to any of the commonly used growth-promoting substances, at least at the concentrations tested. In this connection it might be noted that McGhee and Trager (6) did not find the Harvard synthetic medium to be favorable to the development of *P.*

lophurae in chicken erythrocytes if bovine albumin was substituted for plasma, although they obtained satisfactory parasite multiplication if the synthetic medium was reinforced with plasma. However, Manwell and Brody (10) found the synthetic medium to be incapable of supporting the multiplication of *P. gallinaceum* even when used as a diluent for whole blood.

Effect of Normal Serum.—Both the Harvard group of investigators and Trager found that normal serum or plasma had a beneficial effect as a component of their cultures. A comparison of the relative effects of normal chicken serum and of erythrocyte extract plus the same serum on our system can be

TABLE I

The Effect of a Synthetic Medium as Compared with Erythrocyte Extract on Intact Parasitized Cells

		Phosphorus incorporation, *µg./mg. nucleic acid phosphorus*	
		Synthetic medium	Erythrocyte extract medium
Experiment A	Total nucleic acids	26.9	52.8
Experiment B	PNA	14.6	27.3
	DNA	3.0	17.4

TABLE II

The Effect of Normal Chicken Serum and of Erythrocyte Extract–Serum on Intact Parasitized Cells

	Phosphorus incorporation, *µg./mg. DNA phosphorus*	
	Serum	Erythrocyte extract-serum
PNA	14.3	26.8
DNA	8.7	24.5

seen in Table II. Initial attempts to use serum were discouraging since it produced adverse effects upon the dilute suspensions of parasitized cells employed in our studies. However, it was found possible to eliminate this complication by heating the serum for 1 hour at 56°C. The serum used in this study was obtained without contamination with erythrocyte material by the technique previously described. In the experiment shown 5 per cent cell suspensions were incubated for 20 hours. Except for glucose, no supplementary substances were added to either serum or erythrocyte extract–serum. The extract was used at a concentration of 30 per cent.

The effectiveness of serum relative to a medium containing erythrocyte extract appeared to be somewhat greater than was observed in the case of the

purely synthetic medium. However, the improvement associated with the presence of the extract is readily apparent. The evidence so far suggests that the cell extract possesses certain unique properties in our system for which we have found no obvious substitute.

The question of the extent to which the results obtained were related to the extract itself was considered. Experiments in which the various supplementary substances used in the synthetic medium were omitted from or added to a medium consisting only of the extract prepared with inorganic salts and glucose led to variable results. In some cases a considerable degree of stimulation was obtained by addition of all the supplements while in others the differences between the extract used alone and when fortified with the complete complement were slight. It is our belief that such differences can be attributed to variation in the preparation of the cell extract. In the case of serum, however, increases of up to 35 per cent in the extent of incorporation of phosphorus into DNA were associated with its use as a constituent of the extract medium even when the extract represented an optimal preparation. We consider that an optimal extract is one used at 30 per cent or better and which has been freshly prepared from frozen cells obtained according to the defibrination technique of Trager. Data on the effect of the addition of serum to such an extract are included in a later table and will be omitted here. It is our impression that the combination of such an extract, glucose, and heated serum results in essentially the maximal parasite growth and development possible to achieve in our dilute cell system.

Effect of Fractionation of Erythrocyte Extract by Dialysis.—It is clear from the results presented that the constituent of the medium found to be of primary importance to parasite growth in our dilute cell system was an extract of normal erythrocytes. As such, it would be of interest to determine the nature of its beneficial activity. Table III presents the results of an experiment in which the extract was subjected to dialysis in the cold against redistilled water and both the dialyzed fraction and the dialysate compared with the whole extract and the Harvard type of synthetic medium. The dialysate, prior to use, was concentrated *in vacuo* at low temperature to the volume of extract from which it was derived. All media contained the full complement of supplementary substances used in the synthetic medium, including bovine albumin. The concentration of inorganic phosphorus in all media was adjusted to approximate that of the whole extract, and sterilization was achieved by filtration through Coors porcelain filters. The cells were incubated as 1 per cent suspensions for a period of 18 hours. In this experiment the observations were confined to a study of the whole nucleic acid fraction.

Since the major portion of the beneficial effect of the extract remained with the non-dialyzable portion, this suggests that the extract does not function primarily as a source of readily diffusible growth factors. However, the

presence of active dialyzable substances is indicated by the smaller but definite increase observed with the dialysate as compared with the synthetic medium and by the fact that the stimulation obtained with the two fractions, when added, approximately totals that obtained with the whole extract. Further attempts at fractionation of the extract in terms of its major growth-promoting effect were not successful, the various methods tried yielding poor recovery of total activity and highly variable results. In attempting to explain the nature of the effect of the extract, certain observations may be pertinent: (*a*) other investigators have cultivated erythrocytic forms of plasmodia in the presence of considerably higher concentrations of normal erythrocytes than were used by us (5, 11); (*b*) Trager was successful with more dilute suspension of red cells in the presence of a red cell extract (4); (*c*) Anderson, carrying out continuous cultivation studies on *P. gallinaceum* in these laboratories, found that a high concentration of normal red cells was markedly favorable

TABLE III

The Influence of Dialysis of Erythrocyte Extract upon Its Effect upon Total Nucleic Acid Phosphorus Incorporation in Intact Parasitized Cells

	Synthetic medium	Extract dialysate medium	Dialyzed extract medium	Complete extract medium
Phosphorus incorporation, *μg./mg. nucleic acid P*	26.9	35.5	51.3	62.5
Increase over synthetic medium		+8.6	+24.4	+35.6

to parasite growth (12); (*d*) we have observed that erythrocyte extract is favorable to the maintenance of parasitized cells under conditions in which no growth is expected and that the concentration of the extract required is in inverse relationship to the concentration of red cells present. Our belief is that an excess of normal erythrocytes and an extract of such cells act in a similar and interchangeable manner to affect parasite growth, although how this effect is brought about remains obscure.

The ability to substitute an extract for an excess of normal cells simplifies the system for study, particularly by chemical methods. It is apparent that active diffusible factors can be separated from the extract and that serum contains, in addition, further favorable materials. However, it is also obvious that in such studies it is impossible to divorce effects which are directed upon the host cell and only secondarily involve the parasite from those which are specifically related to the parasite. This problem has, of course, plagued all investigators concerned with intracellular parasites. With a satisfactory method at hand for carrying out studies on the intact parasitized cell, it was decided to attempt comparative studies with lysates to determine whether differential effects could be demonstrated with the two systems.

Studies with Lysates

As was stated in the preceding paper, the possibility of using lysates of parasitized cells was suggested by the work of Speck and his associates (8) in which it was found that lysis of parasitized cells by a specific red cell homolysin-complement system resulted in preparations retaining a considable degree of metabolic activity. Using this technique of lysis, Trager (9) has reported some success in the cultivation of *P. lophurae* in lysates of parasitized duck erythrocytes. We experienced difficulty in obtaining conditions under which any parasite growth could be demonstrated in lysates of chicken erythrocytes parasitized with *P. gallinaceum*, but shortly prior to the termination of our program of research in malaria the technique was developed to the point at which some parasite growth could be obtained consistently, and it became possible to carry out a few comparative studies on intact parasitized cells and on lysates of such cells.

Essential Nature of Erythrocyte Extract.—A variety of methods of handling the lysates and a number of different media preparations were tried before demonstrable parasite growth was realized. During the early phase of this work, for convenience, the radioactivity of the total nucleic acid fraction was followed since no activity was found to be associated with this fraction when obtained from lysates of normal cells after incubation and this had been found to be a reasonable, though less accurate, method of following parasite growth in intact cells. It was found that some preparations showed a continuing increase in the specific activity of the total nucleic acids throughout the 18 to 20 hour period of incubation. However, on separation of the two nucleic acid fractions, no P^{32} could be demonstrated in the DNA and no growth was obvious in stained films, although the parasites were normal in appearance. For work with lysates, the use of the isotope concentration in total nucleic acids or in PNA is completely misleading as an indicator of growth for it is apparently possible to attain maintenance without growth with such preparations over a period of many hours in the presence of almost any preparation of erythrocyte extract. In contrast, in the absence of the extract, the parasites in lysates did not tolerate prolonged incubation and, after a few hours, exhibited marked changes in morphology and staining characteristics. These observations were reflected in the results obtained with P^{32}, with which it was found that the incorporation of the isotope, even into the total nucleic acids, failed to continue beyond a few hours in the absence of the extract, and, in fact, the values at the end of 20 hours were frequently lower than those at 2 to 4 hours, indicative of the subsequent dissolution of material formed early in the course of incubation. Erythrocyte extract thus appears to have a rather fundamental importance to the maintenance of the parasite either in lysates or in intact cells.

Comparison of Parasite Growth in Intact Cells and in Lysates.—The method

which finally produced the greatest parasite growth in lysates utilized a 50 per cent erythrocyte extract prepared as indicated in the section on Methods and Materials. Lysis was carried out in a similar extract, except that it contained no added malate or hexose diphosphate. Even with this medium the DNA phosphorus incorporation values obtained with lysates were considerably lower than those obtained with intact cells, as can be seen in Table IV, which represents a parallel study of the same cells incubated in the intact state and as lysates. The medium for the intact cells was a 30 per cent erythrocyte extract prepared from the same pool of red cells used for the lysate medium and containing 25 per cent heated normal chicken serum. Incubation was carried out for 18 hours.

TABLE IV

The Incorporation of Phosphorus into DNA in Intact Parasitized Cells and in Lysates of the Same Cells

	Phosphorus incorporation, *μg./mg. DNA phosphorus*
Intact cells	21.2
Lysates	3.8

Such a relatively low DNA value as is seen for the lysate would be associated with a generalized retardation of growth of all parasites in an intact cell system. This is *not* the case with lysates, with which excellent growth can be seen in smear, limited, however, to a portion only of the total parasites present. During the development of the medium, it was observed that increases in DNA phosphorus incorporation were associated with demonstrable growth of a larger percentage of the total parasites. Conversely, adverse conditions, such as the use of too heavily parasitized preparations in initiating experiments, resulted in both a decrease in the DNA value and of the number of parasites showing significant growth by smear examination. The medium as developed seemed capable of supporting the growth of only a limited parasite population.

Effect of Malate on Parasite Growth.—The complete medium and technique used by Trager for cultivation studies with lysates of duck erythrocytes parasitized with *P. lophurae* did not prove successful in our studies. However, hexose diphosphate, found by Trager to favor the growth of *lophurae* was likewise slightly stimulatory in our system, although adenosinetriphosphate, also reported favorable to *lophurae*, was without beneficial effect. Malate was the single substance which led to the most striking improvement in the growth of our preparations, either when used alone or combined with hexose diphosphate. The stimulatory effect of malate can be seen in Table V, which compares the DNA phosphorus incorporation in the presence of malate, or of

malate plus hexose diphosphate, with that observed when glucose alone was added to the extract medium. Equimolar sodium and potassium malate were added to the extract diluent to give a concentration of 0.02 M. The medium containing glucose alone was prepared with proportionately more NaCl and KCl to maintain equivalent cation concentrations. The addition of malate can be seen to be associated with an approximately fourfold increase in the DNA values. Since succinate failed to act as a substitute for malate, it would not

TABLE V

The DNA Phosphorus Incorporation in Lysates of Parasitized Cells and the Influence of Malate or of Malate Plus Hexose Diphosphate

	Experiment A		Experiment B	
	Glucose	Glucose + malate	Glucose	Glucose + malate + hexose diphosphate
DNA phosphorus incorporation, *μg./mg. DNA P*...............	0.6	2.4	1.1	3.8

TABLE VI

A Summary of Studies on the Comparative Response of Intact Parasitized Cells and of Lysates to Environmental Variations

	Phosphorus incorporation into DNA, *μg./mg. DNA phosphorus*							
	Control	Serum added	Aerobic	Anaerobic	Control	Quinine	Control	Malonate
Intact cells	8.1	10.9	13.4	0.8	21.0	0	24.5	3.2
Lysates	3.4	3.6	2.4	2.3	3.8	3.1	3.8	2.5

Results represent data from several experiments presented in composite form.

Variations in control DNA values are attributable to variation in initial parasite densities.

seem that the effect of malate can be explained simply by its participation in the citric acid cycle.

Comparative Effect of Environmental Variation on Parasite Growth in Intact Cells and in Lysates.—Although we did not succeed in realizing conditions which would permit the growth of all the parasites in lysate preparations, the development of the method to a point at which it was found to yield consistent growth permitted us to carry out some comparative studies with intact cells. A composite summary of the results of four such studies is given in Table VI. The conditions chosen were ones known or expected to result in rather marked effects on parasite growth in the intact cell and consisted of the addition of an excess of heated serum to the medium, of an anaerobic as compared with an

aerobic environment, and of the addition of the potentially inhibitory substances, malonate and quinine, to the medium. Freshly prepared, concentrated erythrocyte extract media were employed for both intact cells and lysates. Heated normal chicken serum was used at a final concentration of 25 per cent. Anaerobic conditions were produced as described in the section on Methods and Materials. Malonate was used at a final concentration of 0.02 M, being added as the sodium salt to the intact system and as the mixed sodium and potassium salts for the lysates. In both cases, the cation composition of the medium was maintained by a corresponding reduction in the concentration of inorganic sodium and potassium salts used in preparing the media. Quinine was added, as the dihydrochloride, to give a final concentration in the intact preparation of 1×10^{-5} M and in the lysate of 4×10^{-5} M. It has been found (13) that chickens infected with *P. gallinaceum* and treated with therapeutically effective doses of quinine show a quinine concentration in their red cells in the range of 0.5×10^{-5} M to 3×10^{-5} M, and, therefore, the amounts employed by us should be well within the effective range. A lower concentration was employed for the intact cells as a consequence of the observations of Silverman, Ceithaml, and their associates (14) that the concentration of the drug tends to be higher in the erythrocyte than in the surrounding medium.

From the results presented in the table, the responses of lysates to the various conditions can be seen to differ markedly from those of intact cells. As shown in the first section, an increase of some 35 per cent in the DNA phosphorus incorporation of intact cells resulted from the addition of heated serum to the medium, whereas no significant effect, either favorable or unfavorable, was associated with its addition to lysates. Both anaerobiosis and quinine, shown in the second and third sections, proved markedly inhibitory to parasite growth in intact cells; in fact, quinine produced complete inhibition of DNA phosphorus incorporation, a phenomenon not heretofore experienced with intact cells. In contrast, there was no significant inhibition of lysates, either as a result of anaerobic conditions or of the addition of the antimalarial drug. Malonate, alone, as shown in the last section, produced a significant degree of inhibition of both types of preparation, although its effect upon the lysate was considerably less marked than upon the intact cell system.

DISCUSSION

The media and techniques described permit comparative studies of parasite growth to be carried out in a quantitative manner with either intact cells or lysates of such cells. It has been found possible to divide an effective medium into several active fractions and to assess the importance of individual constituents of the medium. It has been recognized that studies on intact parasitized cells may consist primarily of the determination of the conditions necessary for the

maintenance of the host cell in an optimal condition and that information obtained from such studies may bear only indirectly upon the parasite. In an attempt to resolve this problem, comparative studies were carried out upon intact cells and lysates.

With both intact cells and lysates, erythrocyte extract was found to be of major importance as a constituent of the medium, and in both cases improvement in growth was associated with the use of concentrated, freshly prepared extracts. In fact, no growth was obtained in lysates unless the method of handling the cells in preparing the extract was that described by Trager. It seems apparent that the extract has a key relationship to parasite growth in either system.

The lack of an effect of an excess of serum on parasite growth in lysates would suggest that the requirement here was by the host cell or by the host cell–parasite system, rather than by the parasite alone. However, the inclusion of traces of serum with the unwashed cells could render this a quantitative rather than a qualitative difference if the significant constituents are required in much smaller amounts by the lysates than by the intact cell–parasite combination.

The marked depression of parasite growth in intact cells under anaerobic conditions might be related to the observations of a number of investigators that both mammalian and avian erythrocytes parasitized by various species of plasmodia show a vigorous aerobic oxidative metabolism as manifested by a high rate of oxygen consumption. However, the lack of effect of anaerobiosis upon the growth of parasites in lysates is of interest since Speck and his associates (8) found that lysates of erythrocytes parasitized with *P. gallinaceum* likewise possess an active aerobic metabolism. In this connection it might be noted that Anfinsen, *et al.* (2) found that although the simian erythrocyte parasitized with *P. knowlesi* consumes oxygen at a rapid rate, the parasite multiplication observed at very low oxygen tensions was at least as good as that observed at the level of air. These investigators did not report upon the effect of complete anaerobiosis. The difference observed by us in the effect of an anaerobic environment on the intact and lysate preparations leads to the conclusion that the requirement for oxygen is a property of the intact cell. It cannot be said whether the absence of oxygen results in a depression of certain phases of host cell metabolism necessary for parasite growth or whether the conversion of the erythrocyte metabolism to an anaerobic one results in an environment which is detrimental to the parasite.

The striking differential effect observed with quinine is of interest. In line with our previous reasoning, this suggests that the action of the drug is not direct but is mediated by the host cell. As in the case of anaerobiosis, the difference in action on the two systems may be attributable to a blocking effect on host cell metabolism or to the production of changes which render

it an unfavorable environment for the parasite. However, there is a third possibility that the drug as added is not the active agent but that it is in some way activated by the intact cell.

Although malonate is frequently considered to be a specific inhibitor of the succinate oxidase system, its depressant effect upon parasite growth in lysates may or may not imply that this enzyme system is of importance to the parasite. Pardee and Potter (15) found that a high concentration of malonate, such as was used here, inhibits other systems in addition to the succinate one, and they related this to the formation of a complex with magnesium ions. Malonate has also been reported to have some inhibitory effect upon phosphorylation mechanisms (16). The observed differences in the extent of the effect on the intact and lysate preparations may be related to this diversity of activity of the inhibitor, the greater growth inhibition in the intact cell corresponding to inhibition of systems only indirectly related to parasite growth.

In considering the results obtained with the lysate preparations, it should be emphasized that these do not by any means represent isolated parasites in the sense that bacteria grown *in vitro* are isolated. At least potentially, all the enzymes of the host cell are present either in the extract or in the sedimentable fraction added with the parasites. Therefore, it is still not possible to differentiate between direct and indirect effects upon the parasite in an exact sense. However, what probably *is* accomplished is the elimination of the intimate dependence of the parasite on the maintenance of the integrity of the individual host cell's metabolism. With lysates it is quite possible that the red cell extract provides a preformed substitute for certain aspects of the metabolic activity of the intact cell necessary to parasite growth. As such it should be accessible for analysis. It seems reasonable to believe that any effects produced on lysates can be related either to a direct action on the parasite or to host cell systems directly concerned with the parasite and its growth. By utilization of this comparative approach, it may be possible to realize a better understanding of the nature of parasite–host cell relationships and of the mode of action of known and potential chemotherapeutic agents.

SUMMARY

The measurement of the degree of incorporation of P^{32} into nucleic acids has been used as a quantitative means of studying parasite growth and development *in vitro* under various conditions.

An extract of normal chicken erythrocytes was found to be of unique importance as a constituent of the medium, both for intact parasitized cells and for lysates of such cells. Heated normal chicken serum possessed some favorable effect upon intact cells.

The major portion of the beneficial effect of the erythrocyte extract was

found to be associated with the non-dialyzable portion, although the dialysate was shown to possess slight growth-promoting activity. Further attempts to characterize the extract in terms of its favorable effects upon parasite growth were unsuccessful.

A medium and conditions were developed which permitted the growth of a limited parasite population in lysates of parasitized red cells. The most important constituents of the medium were concentrated, freshly prepared erythrocyte extract and malate.

Comparative studies carried out with intact parasitized cells and with lysates showed that substances and conditions exerting quite marked effects upon the intact cell system were without effect or exerted a less marked effect upon parasite growth in lysates. The possible implications of these observations are discussed and the further exploitation of this comparative approach suggested as a means of furthering our knowledge of parasite–host cell relationships.

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