STUDIES ON THE EXTRACELLULAR CULTIVATION OF AN INTRACELLULAR PARASITE (AVIAN MALARIA)

II. The Effects of Malate and of Coenzyme A Concentrates

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Plate 19

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Extracellular survival and development of the erythrocytic stages of the malaria parasite *Plasmodium lophurae* have been obtained *in vitro* in a medium consisting of an extract of duck red blood cells prepared in a special nutrient solution (1). When the erythrocyte extract was further supplemented with adenosinetriphosphate and sodium pyruvate, all or nearly all the extracellular parasites continued their development during the first 2 days of cultivation. By the 3rd day about 15 to 25 per cent of the free parasites seen in stained films appeared degenerate and by the 4th day about 50 per cent.

Out of many changes in the composition of the culture medium which have been tried, two, the addition of potassium malate (2) and the addition of concentrates rich in coenzyme A, have been found to improve the extracellular survival of the parasites.

Methods and Materials

These have been essentially identical with the methods and materials used in the previous work (1).

As before, the erythrocytic stages of *P. lophurae* were obtained from ducklings on the 4th day of their infection. Such ducklings had 40 to 60 parasites per 100 red blood cells, usually with most of the parasites in the uninucleate trophozoite stage. The parasites were liberated from their host cells by the same immune serum hemolytic system previously used, except that the mixture consisted of 2 ml. of 20 per cent red cell suspension in erythrocyte extract, 1.6 ml. of additional erythrocyte extract, 0.07 ml. of guinea pig serum, and 0.4 ml. of anti-duck erythrocyte serum from rabbits.

The Culture Media.—The duck erythrocyte extract was always prepared in the gelatin type of nutrient solution of the composition shown in Table I of the first paper of this series (1). It was supplemented with yeast adenylic acid and cozymase to give concentrations of 1.4 and 0.15 millimols per liter respectively. During the course of the experiments with malic acid, these two supplements were prepared in the manner previously described. After the favorable effect of malic acid had been established, they were combined with the malic acid into a single supplement prepared in the following way:

500 mg. of yeast adenylic acid was suspended in 10 ml. of water and dissolved by the dropwise addition of 1 N NaOH to a pH of 5.8. 800 mg. of *l*-malic acid was added and the pH readjusted to 5.8 by the addition of slightly over 2 ml. of 5 N KOH. 100 mg. of cozymase

(65 per cent) was then added and the solution was diluted to 20 ml. It was sterilized by filtration through an ultrafine glass filter, tubed, frozen, and stored in a deep freeze. 0.2 ml. of solution was added to each 10 ml. of completed red cell extract medium to give final concentrations, in millimols per liter, of adenylic acid 1.4, *l*-malic acid 6.0, and cozymase 0.15. A similar supplement, but without malate, was also used in some experiments.

Each experimental flask received initially, and with each addition of fresh culture medium, 0.1 ml. of a supplement of adenosinetriphosphate with sodium pyruvate prepared as previously described.

Potassium malate solution (0.4 M) was prepared by dissolving 2.68 gm. of *l*-malic acid in water, neutralizing to pH 6.7 with 5 N KOH, and diluting to 50 ml. The solution was sterilized by filtration through a Selas 03 porcelain filter. 0.05 to 0.1 ml. of this was added to each flask at the start of the experiment and with each change of culture medium, to give concentrations of about 0.005 to 0.01 M. 0.6 M potassium fumarate and potassium succinate solutions were prepared in a similar way and added at the rate of 0.05 to 0.1 ml. per 10 ml. of complete red cell extract to give concentrations of 0.003 to 0.006 M. An autoclaved 1.5 M solution of sodium citrate was used similarly to give a concentration of 0.015 M.

Several different concentrates of coenzyme A have been used. The first [CoA(a)] was obtained through the courtesy of Dr. W. Maas of the United States Public Health Service Tuberculosis Research Laboratory. It had a potency of 30 Lipmann units (3) per mg. It was made up in a solution of 1 mg. per ml. water and added to each culture flask with each addition of medium at the rate of 0.1 ml. per 3.5 ml. total, giving a concentration of less than 1 unit per ml. The second type of concentrate [CoA(1)] was a crude "liver coenzyme concentrate" purchased from Armour & Co. and stated to have approximately 10 units coenzyme A activity per mg. 200 mg. was suspended in 20 ml. of 0.15 M phosphate buffer of pH 7.4. Most of the material dissolved to give a solution of pH 5.1. This was brought to pH 6.5 with a few drops of 0.5 N KOH. The small amount of undissolved material was removed by centrifugation. The clear yellow supernatant, like all the other coenzyme A solutions, was sterilized by filtration. 0.2 to 0.4 ml. was added to each flask with each addition of culture medium, giving concentrations of 6 to 12 units per ml.

Two more highly purified concentrates of coenzyme A were kindly sent me by Dr. J. D. Gregory and Dr. F. Lipmann of Harvard University. One [CoA(2)] had a potency of 110 units per mg. 11 mg. was dissolved in 10 ml. of 0.15 M phosphate buffer giving a solution with a pH of 7.2 This was adjusted to pH 6.3 with 0.1 N HCl and the volume made to 11 ml. 0.2 ml. was added to each flask with each addition of medium, giving a concentration of 6 units per ml. The other [CoA(3)] had a potency of 170 units per mg. 12 mg. was dissolved in 12 ml. of water, giving a solution with a pH of 6.2. This was used at the rate of 0.15 ml. per flask to give a concentration of slightly over 6 units per ml.

A triphosphopyridine nucleotide preparation was purchased from the Sigma Chemical Co. 20 mg. of the TPN "80" was dissolved in 5 ml. of water and sterilized by filtration. It was added at the rate of 0.15 ml. per flask to give a concentration of about 0.0002 M.

Preparation of the Cultures.—The culture vessels were, as before, 50 ml. Erlenmeyer flasks equipped for the passage of a gas mixture. Each flask received typically 3 ml. of erythrocyte extract (already containing yeast adenylic acid and cozymase, and in the later experiments *l*-malic acid) and 0.1 ml. of adenosinetriphosphate-sodium pyruvate supplement. If other supplements were to be added, the volume of erythrocyte extract was proportionately reduced. The total volume of additional supplements was usually 0.2 ml. and never exceeded 0.4 ml. The flask was then inoculated with 0.3 ml. of the suspension of hemolyzed red cells and free parasites. The flasks were incubated on a rocker at 39–40°C. and a slow current of 95 per cent air with 5 per cent carbon dioxide was passed over the surface of the medium. The culture fluid was changed after the first 18 hours and thereafter at approximately 12 hour intervals. The fresh culture medium consisted of 3.4 ml. of appropriate erythrocyte

extract (or porportionately less if additional supplements were to be used), 0.1 ml. of adenosinetriphosphate-sodium pyruvate supplement, and the required amounts of other supplements.

The changing of the culture medium with a minimum of disturbance to the parasites depends on the accumulation of the hemolyzed red cells and free parasites in a scum on the wall of the flask during the first 18 hours of incubation. When the experiments previously described (1) were conducted at the Rockefeller Institute laboratories at Princeton, New Jersey, only rarely would a flask be encountered in which the scum failed to form in adequate amounts. When the work was moved to the Rockefeller Institute in New York, entire sets of flasks would show little or no scum formation. In such experiments the first change of culture fluid could be effected only by centrifuging down the parasites and hemolyzed red cells and resuspending them in fresh medium. A scum would then form within the following 12 hours so that further changes of culture medium could be performed in the usual way. The single centrifugation invariably resulted in larger than usual proportions of degenerate parasites on the 2nd and 3rd days. Experiments in which centrifugation was necessary have not been included in the results reported in this paper, except for a single example (Table II, Experiment F). The difficulty with scum formation persisted in spite of special precautions with the cleaning of glassware and in spite of discontinuance of the use of cotton plugs during the sterilization of the flasks. It has been largely overcome by lining the lower portion of the wall of the culture flask with a thin layer of clotted normal duck plasma. In order to do this, blood was drawn without anticoagulant from the neck vein of a duckling 3 to 4 weeks old. The blood was centrifuged and the plasma drawn off. 0.2 to 0.25 ml. of plasma was placed in each empty culture flask. Each flask then received individually 0.01 to 0.05 ml. of a chick embryo extract (one 10 day chick embryo ground in a glass tissue grinder in 4 ml. of an isotonic glucose-salt solution and centrifuged). The flask was held on its side and rotated rapidly until clotting occurred. The culture media and parasites were then introduced. In flasks so prepared, an adequate scum usually formed within 18 hours. This method had the further advantage of maintaining the original soft consistency of the scum even through the 3rd and 4th days of cultivation. As long as scum formation was comparable, the extracellular survival of the parasites was not affected by the presence of the plasma clot lining.

Criteria for Judging the Survival and Development of the Extracellular Parasites.—The morphological criteria used have been fully discussed in the previous work.

In fresh preparations examined with dark contrast phase microscopy, the three types of abnormal appearance of the cytoplasm have been again observed (too dark, too bright, and patchy). Fig. 3 shows the opaque, bright appearance, not previously illustrated, which was of special interest in connection with the effect of malic acid. This is to be contrasted with the translucent, medium bright appearance of healthy extracellular parasites (Figs. 1 and 2).

In the films stained with Giemsa stain, counts were made of the numbers of degenerate parasites and of the various stages of the parasites of normal or approximately normal appearance. Comparison of the latter counts with the similar counts made on the initial hemolyzed preparation gave an indication of the degree of nuclear development. Usually there was good agreement between counts made at different times from the same slide or from separate slides prepared at the same time from the same flask (see, for example, Table II, Experiment G, flasks 6 and 7 on the 3rd day). Indeed, the different flasks of an experiment showed at first much the same course of events, differences becoming evident generally not until the 3rd day.

RESULTS

The conditions which have been used permitted uniformly good survival and development of the extracellular parasites through the 2nd day (Fig. 2).

TABLE I

The Effect of Malic Acid and Related Compounds on the Extracellular Development of P. lophurae as Seen in Stained Films

200 parasites in successive microscopic fields were counted and classified.

Experiment	Preparation	Time *in vitro*	Medium	No. of parasites of normal appearance						No. of degenerate parasites
				Very young	Uninucleate trophozoites	2 to 4 nuclei	Over 4 nuclei	Gametocytes	Total	
		days								
A	Original	0	—	1	192	6	1	0	200	0
	Flask 1	2	Standard* without malate	0	139	47	3	0	189	11
	2			2	167	17	3	0	189	11
	3		Standard + malate (sample	1	129	56	2	1	189	11
	4		A) 0.006 M	2	138	43	3	1	187	13
	5		Standard + malate (sample	5	133	50	2	0	190	10
	6		B) 0.006 M	2	140	42	7	0	191	9
	Flask 1	3	Standard* without malate	0	129	34	1	0	164	36
	2			1	142	31	1	0	175	25
	3		Standard + malate (sample	0	121	39	5	1	166	34
	4		A) 0.006 M	0	97	62	5	2	166	34
	5		Standard + malate (sample	0	111	64	4	1	180	20
	6		B) 0.006 M	0	115	57	2	1	175	25
B	Original	0	—	9	118	60	13	0	200	0
	Flask 1	2	Standard* + malate 0.006 M	56	81	26	30	0	193	7
	2			27	109	25	31	0	192	8
	3		Standard + malate 0.006 M	85	54	25	26	0	190	10
	4		+ fumarate 0.006 M	36	93	29	30	2	190	10
	5		Standard + malate 0.006 M	33	122	18	25	0	198	2
	6		+ fumarate 0.003 M + succinate 0.003 M	51	75	30	36	0	192	8
	Flask 1	3	Standard + malate 0.006 M	67	53	12	9	0	141	59
	2			63	61	17	10	0	151	49
	3		Standard + malate 0.006 M	59	48	18	2	0	127	73
	4		+ fumarate 0.006 M	45	47	10	4	0	106	94
	5		Standard + malate 0.006 M +	44	68	14	6	0	132	68
	6		fumarate 0.003 M + succinate 0.003 M	57	66	14	3	0	140	60

TABLE I—*Concluded*

Experiment	Preparation	Time *in vitro*	Medium	No. of parasites of normal appearance						No. of degenerate parasites
				Very young	Uninucleate trophozoites	2 to 4 nuclei	Over 4 nuclei	Gametocytes	Total	
		days								
C	Original	0	—	0	177	19	4	0	200	0
	Flask 1	3	Standard* + malate 0.006 M	21	105	25	16	0	167	33
	2			12	105	30	14	4	165	35
	3		Standard + malate 0.006 M	26	109	31	11	0	177	23
	4		+ citrate 0.015 M	23	97	15	10	1	146	54
	5		Standard + malate 0.006 M	9	78	25	8	0	120	80
	6		+ citrate 0.015 M + fumarate 0.003 M	5	84	37	9	1	136	64
	7		Standard + malate 0.006 M	25	82	44	11	1	163	37
	8		+ citrate 0.015 M + succinate 0.003 M	15	88	51	17	2	173	27

* Standard signifies the red cell extract medium prepared as previously described (1) in nutrient solution with gelatin and supplemented with cozymase, yeast adenylic acid, sodium pyruvate, and adenosinetriphosphate.

When the initial hemolyzed suspension contained almost exclusively young uninucleate trophozoites, there occurred within 18 hours a marked increase in the proportion of trophozoites with two to four nuclei. This increase usually continued to the 2nd day and was accompanied by a moderate increase in the proportions of later segmenters (over four nuclei). There was no accumulation of very young forms, such as would occur if the merozoites which were being formed were unable to develop further, and only 5 per cent or less of the parasites were degenerate (see tables I and II, Experiments A, E, G, and I). If the initial hemolyzed suspension contained a considerable proportion of parasites with two to four nuclei (see Table I, Experiment B), the proportion of such forms generally decreased by the 1st day, while the proportion of late segmenters and the very young forms increased. These changes progressed further in the same direction on the 2nd day. By the 3rd day such experiments generally showed somewhat more degenerate parasites and a greater accumulation of very young forms than experiments begun with most of the parasites in the uninucleate trophozoite stage.

The effects of malic acid and coenzyme A concentrate became evident

TABLE II

The Effect of Coenzyme A Concentrates on the Extracellular Development of P. lophurae as Seen in Stained Films

200 parasites in successive microscopic fields were counted and classified.

Experiment	Preparation	Time *in vitro*	Medium	No. of parasites of normal appearance						No. of degenerate parasites
				Very young	Uninucleate trophozoites	2 to 4 nuclei	Over 4 nuclei	Gametocytes	Total	
		days								
D	Original	0	—	6	179	12	1	0	198	2
	Flask 1	3	Standard* + malate 0.006 M	8	118	38	13	3	180	20
	2			3	128	40	6	1	178	22
	3		Standard + malate 0.006 M	15	131	32	12	0	190	10
	4		+ CoA(1)‡ 6 units/ml.	9	138	27	14	2	190	10
E	Original	0	—	6	179	6	6	1	198	2
	Flask 1	2	Standard* + malate 0.006 M	2	151	30	11	1	195	5
	2			1	164	22	5	3	196	4
	3		Standard + malate 0.006 M	1	159	31	5	1	197	3
	4		+ CoA(1) 6 units/ml.	1	167	21	7	3	199	1
	5		Standard + malate 0.006 M + CoA(1) 12 units/ml.	2	164	28	2	2	198	2
	Flask 1	3	Standard* + malate 0.006 M	5	133	19	0	1	158	42
	2			0	142	22	7	2	173	27
	3		Standard + malate 0.006 M	6	155	24	2	1	188	12
	4		+ CoA(1) 6 units/ml.	1	163	21	1	0	186	14
	5		Standard + malate 0.006 M + CoA(1) 12 units/ml.	0	164	18	5	1	188	12
F	Original	0	—	13	180	6	1	0	200	0
	Flask 1	2	Standard* + malate 0.006 M	0	93	65	27	0	185	15
	2			4	85	58	38	0	185	15
	3		Standard + malate 0.006 M	0	88	73	33	0	194	6
	4		+ CoA(1) 6 units/ml.	3	87	77	26	2	195	5
G	Original	0	—	2	190	6	2	0	200	0
	Flask 1	3	Standard* + malate 0.006 M	6	136	28	8	2	180	20
	2			2	131	44	5	0	182	18

TABLE II—*Continued*

Experiment	Preparation	Time *in vitro*	Medium	No. of parasites of normal appearance						No. of degenerate parasites
				Very young	Uninucleate trophozoites	2 to 4 nuclei	Over 4 nuclei	Gametocytes	Total	
		days								
	3		Standard + malate 0.006 M	2	148	27	9	2	188	12
	4		+ CoA(1) 6 units/ml.	0	142	41	3	0	186	14
	5			0	159	26	2	1	188	12
	6		Standard + malate 0.006 M	1	157	27	2	1	188	12
			+ CoA(2) 6 units/ml.	1§	148	31	6	0	186	14
	7		Standard + malate 0.006 M	3	138	45	1	0	187	13
			+ CoA(2) 6 units/ml.	7‖	128	48	5	1	189	11
H	Original	0	—	2	192	4	2	0	200	0
	Flask 1	3	Standard* + malate 0.006 M	5	94	49	17	3	168	32
	2		Standard + malate 0.006 M + CoA(1) 6 units/ml.	0	118	49	12	1	180	20
	3		Standard + malate 0.006 M	9	100	53	18	0	180	20
	4		+ CoA(3) 6 units/ml.	3	120	53	7	0	183	17
	5		Standard + malate 0.006 M	2	117	47	6	0	172	28
	6			3	107	58	4	2	174	26
I	Original	0	—	1	188	7	2	0	198	2
	Flask 1	2	Standard* + malate 0.006 M	2	131	47	13	1	194	6
	2			3	141	40	10	1	195	5
	3		Standard + malate 0.006 M	6	101	58	29	1	195	5
	4		+ CoA(3) 6 units/ml.	0	137	50	9	1	197	3
	5		Standard + malate 0.006 M	8	128	46	12	0	194	6
	6		+ TPN 0.0002 M	4	147	34	10	0	195	5
	7		Standard + malate 0.006 M	3	151	32	13	0	199	1
	8		+TPN 0.0002 M + CoA(3) 6 units/ml.	4	130	46	16	1	197	3

TABLE II—*Concluded*

Experiment	Preparation	Time *in vitro*	Medium	No. of parasites of normal appearance						No. of degenerate parasites
				Very young	Uninucleate trophozoites	2 to 4 nuclei	Over 4 nuclei	Gametocytes	Total	
		days								
	Flask 1	3	Standard* + malate 0.006 M	3	102	57	17	0	179	21
	2			2	107	44	13	0	166	34
	3		Standard + malate 0.006 M	6	136	25	18	1	186	14
	4		+ CoA(3) 6 units/ml.	4	132	43	11	1	191	9
	5		Standard + malate 0.006 M	12	114	41	11	0	178	22
	6		+ TPN 0.0002 M	6	122	32	14	1	175	25
	7		Standard + malate 0.006 M	6	127	47	4	0	184	16
	8		+TPN 0.0002 M + CoA(3) 6 units/ml.	5	111	55	15	0	186	14

* See footnote to Table I.

‡ CoA(1), (2), (3) see text under Methods and Materials.

§ The figures on this line were obtained from a duplicate slide prepared from flask 6.

‖ The figures on this line were obtained from a duplicate slide prepared from flask 7.

chiefly on the 3rd day of cultivation. With both of these materials added to the medium, the condition of the extracellular parasites was nearly as good on the 3rd day as on the 2nd day (Fig. 2, Table II). More extensive degeneration set in on the 4th day, but in some cultures kept to the 4th day, a majority of the parasites were still of healthy appearance.

The Effects of l-Malic Acid and of Certain Other Related Compounds.—In a series of seven experiments performed at different times, in somewhat different ways, and with two different samples of *l*-malic acid, the flasks which contained red cell extract supplemented with potassium malate invariably showed, on the 3rd day, parasites of better appearance, as seen with phase contrast in fresh preparations, than the control flasks. On the 3rd day, flasks without malate usually had among the degenerate parasites a considerable proportion of the conspicuous, opaque, overbright forms. Very few such forms could be found in the flasks containing malate. Moreover, the latter flasks had fewer degenerate parasites of all types. This was especially true when the malate was supplied at 0.005 M rather than at 0.01 M. In one experiment in which malate was added at only 0.0005 M, no effect was noted. In spite of the consistently favorable effect of the higher concentrations of malate

on the appearance of the extracellular parasites in fresh preparations, no effect could be detected in stained films (Table I, Experiment A).

When fumarate was added to a medium containing malate, survival of the parasites was definitely worse than in the absence of both malate and fumarate. This deleterious effect of fumarate at 0.003 to 0.006 M was evident in stained films on the 3rd day of cultivation as well as in the fresh preparations on both the 2nd and 3rd days (Table I, Experiment B). The effect was evident in the presence of citrate, which itself had no effect (Table I, Experiment C). Succinate likewise had no effect, either in the presence of fumarate or citrate (Table I, Experiments B and C).

The Effect of Coenzyme A Concentrates.—The first preparation available [CoA(a)] was tested only at the relatively low concentration of somewhat under 1 Lipmann unit per ml. In one or two experiments, a slight favorable effect was noted, but this was not consistently obtained. However, with the "liver coenzyme concentrate" [CoA(1)] supplied at a concentration sufficient to furnish about 6 units per ml., definite favorable effects have been observed in every experiment in which the material has been tested. These effects were evident on the 3rd day of cultivation both in the better appearance of the parasites in fresh preparations and in the smaller number of degenerate parasites counted in the stained films (Table II, Experiments D, E, G, and H). The best extracellular parasites so far seen after 4 days *in vitro* were observed in fresh preparations from flasks containing the coenzyme A concentrate, but at this time the proportion of degenerate parasites counted in stained films was the same as in control flasks without coenzyme A. Experiment F (Table II) shows an effect of the coenzyme A concentrate evident on the 2nd day in an experiment in which it was necessary to subject all the parasites to the somewhat harmful process of centrifugation after the first 18 hours of incubation.

The two more highly purified preparations of coenzyme A [CoA(2) and CoA(3)] gave results very similar to those obtained with the liver coenzyme concentrate (Table II, Experiments G, H, and I). The further addition, in two experiments, of the triphosphopyridinenucleotide appeared to have no effect (Table II, Experiment I).

DISCUSSION

The experimental results just presented indicate that *P. lophurae* requires, for maintenance outside of its host cell, *l*-malic acid and probably coenzyme A. These are in addition to the known factors demonstrated in the previous work and to the possibly numerous unknown factors present in the erythrocyte extract. The apparent need of the parasites for an external source of coenzyme A as well as of adenosinetriphosphate, two substances considered to be of

prime importance in biosynthetic mechanisms (4), emphasizes how far they have gone along the road of parasitic degeneration.

Experiments on the respiration of surviving preparations of free malaria parasites have demonstrated the utilization by the parasites of succinate and fumarate as well as of malate (5, 6). It is therefore of considerable interest that of these three closely related compounds, only malate had a favorable effect on the prolonged extracellular survival of *P. lophurae*. Clarke (7) has recently shown an even more striking favorable action of malate, but not of succinate, on the extracellular survival of *P. gallinaceum* during 18 hours *in vitro*.

The favorable effect of concentrates rich in coenzyme A is not altogether surprising. The survival of *P. lophurae* in duck erythrocyte suspensions maintained *in vitro* was early found to be prolonged in the presence of calcium pantothenate (8). This finding was followed by the demonstration that pantothenic acid deficiency inhibits the development of *P. gallinaceum* in the intact host animal (9) and that certain analogues of pantothenic acid are effective antimalarial drugs (10). For a more detailed elucidation of the role of coenzyme A in the metabolism of malaria parasites further experiments will be required with more highly purified preparations and with media from which calcium pantothenate has been omitted.

SUMMARY

The extracellular survival and development *in vitro* of the erythrocytic stages of *Plasmodium lophurae* were favored by the addition to the culture medium of *l*-malic acid and concentrates rich in coenzyme A. In a concentrated extract of duck erythrocytes supplemented with these two substances in addition to adenosinetriphosphate, sodium pyruvate, and certain other materials of like nature, only 5 to 10 per cent of the extracellular parasites had become abnormal after 3 days of cultivation.

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EXPLANATION OF PLATES

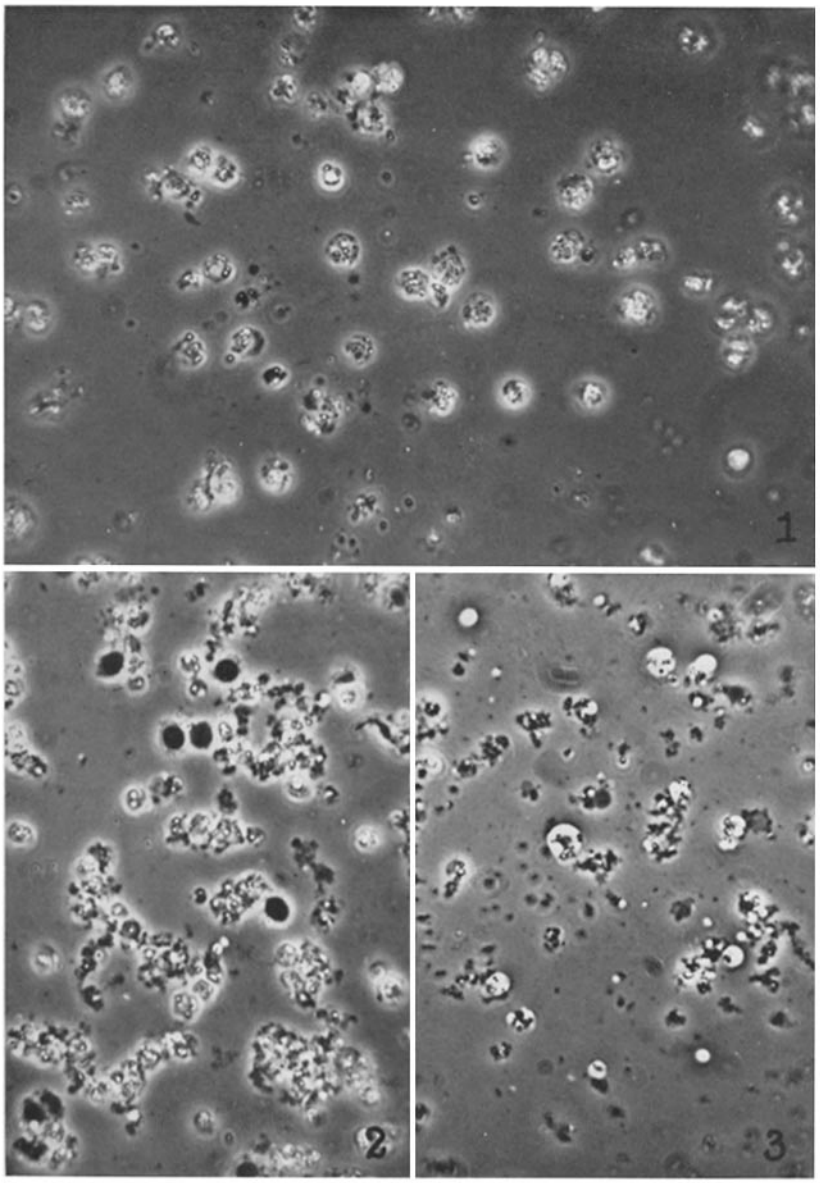
EXPLANATION OF PLATE 19

Photomicrographs of fresh preparations of *P. lophurae* developing extracellularly. Phase contrast. ×776.

FIG. 1. After 2 days in culture in the standard medium (see footnote, Table I) with 0.006 M malate. Note the smooth, medium bright appearance of the cytoplasm of most of the parasites. One large parasite (near the center) has completed segmentation and the individual merozoites may be readily seen.

FIG. 2. After 3 days in culture in the standard medium with 0.006 M malate and a purified coenzyme A concentrate [CoA(2)] supplying 6 units per ml. Although some obscuring granular material is present, most of the parasites have the same appearance as in Fig. 1.

FIG. 3. After 2 days in a culture in standard medium which had been subjected to too violent shaking during the hemolysis of the infected red cells. Only one small trophozoite (at the left center) has normal cytoplasm. All the others show the opaque bright type of degeneration, which was seen, but to a lesser extent and chiefly on the 3rd day, in ordinary cultures in standard medium without malate.



(Trager: Extracellular cultivation of intracellular parasite. II)