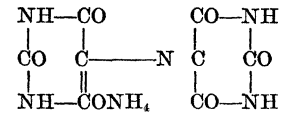
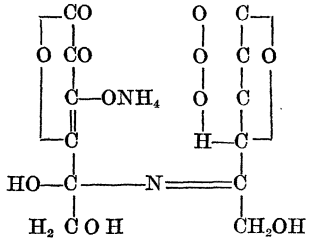
(2) Murexide (Slimmer and Stieglitz formula):



(3) Ascorbic acid (proposed formula):



In support of the theory, it may be argued that in both the ninhydrine and the present test only compounds containing amino group α to a carboxyl are reacting and that we found that in the murexide test the customary reagent, NH_3 water, may be substituted by α-amino compounds (dl-alanine). Also, if dehydroascorbic acid is tested directly with ammonia water and heat, a red color develops without the aid of an oxidizing agent. The facts that the test is more delicate if performed in glacial acetic acid (non-aqueous) media and that copper may be substituted for hydrogen peroxide in the test, indicate that ascorbic acid must be first converted to dehydroascorbic acid to react with $-NH_2$ to yield a colored compound.

It may be stated that the suggestion of Harding and MacLean[4] that 0.2 cc of pyridine to be used for increasing the sensitivity of the ninhydrine test is not applicable to the ascorbic acid reaction because pyridine, along with some other compounds, yields a yellow color in the above procedure which obscures the faint red colors developed.

Conclusions: A new reaction and preliminary colorimetric test of distinctive specificity has been developed by using dl-alanine and H_2O_2 as reagents for the detection of ascorbic acid. It is probable that the color develops with dehydroascorbic acid in a manner similar to the ninhydrine or murexide tests. While the new test may not be fully applicable at the present time to determine ascorbic acid in animal and human materials, it may be used to test the identity of ascorbic acid in official (*e.g.,* U.S.P.) preparations.

We are greatly indebted to Dr. J. H. Roe for checking the reaction and for his encouragement to continue this research.

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[4] V. J. Harding and R. M. MacLean, *Jour. Biol. Chem.,* 20: 217, 1915.

IN VITRO GROWTH AND MULTIPLICATION OF THE MALARIA PARASITE, PLASMODIUM KNOWLESI[1, 2]

WHETHER one is considering the biochemical, biological, immunological or chemotherapeutic aspects of the malaria problem, questions arise which could be more readily answered by experimentation on parasites grown *in vitro* than on those sheltered by the host. This well-recognized fact has led many previous investigators to attempt the *in vitro* cultivation of the malaria parasite. Undoubtedly, the most successful and best-documented work to date on the erythrocytic form has been that of Trager,[3, 4] who clearly demonstrated that the bird malaria parasite, *P. lophurae,* would survive *in vitro* up to 16 days at body temperature. Never did Trager observe an increase *in vitro* in the total number of parasites comparable to that seen in the host. The parasite population in his experiments usually remained constant for the first few days and then declined. He concluded that, though development must be continuing in such preparations, the death rate, particularly after the first few days, far exceeds the birth rate.

We wish to report here a brief summary of the results obtained during the past year on the *in vitro* cultivation of the erythrocytic form of the malaria parasite, *P. knowlesi,* in which we have regularly observed growth and good multiplication. *P. knowlesi* was chosen for our work because this parasite has a 24-hour cycle, produces a heavy infection of red cells in the monkey, *Macaca mulatta,* and will also produce infection in man. Moreover, since our chief interest lay in making biochemical and metabolic studies on the parasite, we preferred to deal with a host possessing a non-nucleated red blood cell.

Two types of techniques have been developed. One, which we have termed the rocker-dilution, consists of the dilution of 1 part of whole blood with 3 parts of a nutrient fluid whose composition is given in Table 1. This mixture, usually 6.0 ml in total volume though 50 ml have been used, is placed in a tube or flask equipped with gas inlet and outlet tubes. The container is then placed on a rocking machine which just keeps the red cells in gentle motion. A slow flow of 5 per cent. CO_2: 95 per cent. air is passed into the vessel without being allowed to bubble through the liquid. The whole procedure is carried out under sterile conditions and the cultivation performed at

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[2] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College.

[3] W. Trager, *Jour. Exp. Med.,* 74: 441, 1941.

[4] W. Trager, *Jour. Exp. Med.,* 77: 411, 1943.

TABLE 1

COMPONENTS OF NUTRIENT MEDIUM*

	gm/L		microgm/L
$MgCl_2$	.095	Adenine Sulfate	250
$CaCl_2$	.056	Guanine-HCl	250
KCl	.410	Thymine	125
NaCl	5.825	Xanthine	250
Na_2HPO_4	.301	Uracil	250
$NaHCO_3$†	2.35	Ascorbic Acid	5000
Glucose	2.50	Biotin	16
Difco Proteose Peptone	1.50	Choline	500
Stearns Amino Acids‡	0.50	Cocarboxylase	400
Glycerol	0.25	Nicotinic Acid	1000
Sodium Acetate	0.15	Nicotinamide	1000
		d-Ca Pantothenate	500
		Pyridoxine	500
		Ribose	500
		Riboflavin	500
		Thiamine	1000

* The freezing point of this medium is $-0.60°$ C. ± .02, and the pH after equilibration with 5 per cent. CO_2: 95 per cent. air is 7.45 ± 0.1.
† Added as Na_2CO_3 and converted to bicarbonate by passing CO_2 gas through the solution.
‡ Fortified with glycine and histidine.

a temperature of 38.5° C. Best results have been obtained when the number of total parasitized cells in the initial mixture is limited to not more than 25,000 per cu.mm., and the total number of red blood cells to 1.25×10^6 per cu.mm. This is usually accomplished by diluting highly parasitized blood with several times its volume of normal blood in order to ensure the presence of an adequate amount of normal blood constituents in the final mixture.

The other technique, which has been termed the rocker-perfusion, employs a Cellophane membrane to separate whole parasitized blood from the nutrient medium. Several types of apparatus for this purpose have been developed. For volumes of blood approximating 1 ml, type 1 is used in which a Cellophane membrane is stretched over one end of a glass tube 30 mm in diameter, and the blood placed within the tube directly upon the Cellophane membrane. This tube is mounted in a vessel containing about 30 ml of nutrient fluid in such a way that the membrane end is just wetted by the nutrient fluid. For volumes of blood approximating 15 ml, type 2 is used in which a coil of Cellophane tubing (8/32 in. dia.) supported by a glass form is immersed in the blood and nutrient fluid allowed to flow through the Cellophane tubing from a reservoir at a rate of approximately 1,500 ml per 24 hours. In both types of apparatus, the container is rocked with a slow stream of 5 per cent. CO_2: 95 per cent. air passing through it. All operations are performed under sterile conditions and cultivation carried out at 38.5° C. As in the rocker-dilution technique, we have usually employed parasitized blood diluted with normal.

Cultivation with all methods is usually carried on for a period of 24 hours. If subculture is performed, then a fresh setup is made each 24 hours, the cultivated parasitized blood being diluted with normal whole blood and the nutrient fluid replaced by fresh.

A summary of the results obtained with the various techniques is given in Table 2. In the 89 experiments given here, the average increase in parasite count within 24 hours has been three- to fourfold. In some sixty monkeys studied to date, the average increase *in vivo* for a similar period and at comparable percentages of parasitization has also been of this order of magnitude.

TABLE 2

SUMMARY OF IN VITRO CULTIVATION EXPERIMENTS IN WHICH TWOFOLD OR BETTER MULTIPLICATION OF PARASITES HAS OCCURRED IN 20–24 HOURS

Method	Total expts.	Number of experiments with the multiplication listed					Avg. multiplication
		2 fold	3 fold	4 fold	5 fold	6–8 fold	
Rocker Dilution	23	2	5	8	5	3	4.0
Rocker Perfusion Type 1	22	3	9	3	3	4	4.0
Rocker Perfusion Type 2	44	19	8	10	4	3	3.2

TABLE 3

DETAILED COUNT ON CULTIVATION EXPERIMENTS RUN BY BOTH TECHNIQUES

Time (hrs.)	0		5		10		24	
Technique*	R.D.	R.P.	R.D.	R.P.	R.D.	R.P.	R.D.	R.P.
Red cells/ cmm × 10^6	1.32	3.98	1.30	4.07	..	4.04	1.10	3.78
Parasite count, per cent.	1.8	2.0	2.0	2.2	..	9.2	7.4	9.6
Differential Distribution								
Rings, per cent.	1	1	1	2	..	61	3	12
Trophozoites, per cent.	96	83	29	38	..	31	95	80
Schizonts, per cent.	3	14	69	52	..	3	0	1
Segmenters, per cent.	0	1	0	6	..	2	0	0
Gametocytes, per cent.	0	1	1	2	..	1	2	4
Degenerate and unrecognizable, per cent.	0	0	0	0	..	2	0	3

* R.D. = Rocker Dilution. R.P. = Rocker Perfusion, Type 2. The blood for these two experiments came from two different monkeys so that the similarity in count is merely fortuitous.

In Table 3, a complete differential count for one experiment run by each technique is given. The shift in distribution of the various forms with time is clearly evident, segmentation having occurred sometime between the fifth and tenth hour of *in vitro* cultivation. At the end of 24 hours, the distribution of forms is about the same as at the start of the experiment though four to five times as many parasites are then present. Differential counts for all the 89 experiments summarized in Table 2 are also on record and show a similar picture.

Using the rocker-dilution technique, three successive generations of *P. knowlesi* have been grown *in vitro,* while with the rocker-perfusion (type 1)

technique six successive generations have been obtained. In the latter case, the infectivity of the blood after six days' culture *in vitro* was demonstrated by inoculation into a monkey. It should be pointed out, however, that in order to obtain subculture with the rocker-perfusion technique, it is necessary to replace about one fourth of the nutrient medium with blood serum. Indeed, we have recently found that better *in vitro* multiplication and growth result during the first 24 hours by the perfusion technique if some serum is present in the nutrient medium. This fact emphasizes one of the basic reasons for our employment of two types of cultivation techniques. We find that the perfusion technique is particularly useful for the study of the nutrient requirements of the parasites, because any low molecular weight material essential for growth and not present in the nutrient fluid will be dialyzed away from the parasite and its deficiency rapidly made apparent. In the rocker-dilution technique, it is obvious that such deficiencies in the nutrient media will not be so readily observable. However, the rocker-dilution technique, because of its simplicity and lack of a Cellophane membrane, has proved very useful in testing the action of antimalarial drugs, immune serum, etc., on the growth of the malaria parasite *in vitro*.

Chemical and metabolic studies have also been made on parasites grown *in vivo* and *in vitro*. Increases have been observed in the content of fatty acids, flavine adenine dinucleotide, total phosphorus, 15-minute hydrolyzable phosphorus, phospholipid phosphorus and nucleic acid phosphorus (by difference) in the red blood cells as their parasite count increases either *in vitro* or *in vivo*. Similar studies on glucose and oxygen consumption and lactate production have also been carried out. We have encountered a striking difference between *in vitro* and *in vivo* grown parasites only in their oxygen consumption. Multiplication of parasites *in vitro* has not been attended by the same increase in oxygen consumption that is observed during multiplication *in vivo*. We have as yet no explanation for this phenomenon, but it may be a reflection of some deficiency or toxic agent in our media or an indication that, if the parasite is well supplied with nutrients, it can exist largely on energy derived from anaerobic processes. In support of the latter explanation is our finding that a gas phase low in O_2 (0.37 per cent. O_2: 5 per cent. CO_2: 94.63 per cent. N_2) permits at least as good growth and multiplication *in vitro* as in 95 per cent. air: 5 per cent. CO_2. Definitely detrimental to *in vitro* growth is a gas phase high in oxygen content (95 per cent. O_2: 5 per cent. CO_2).

The composition of the nutrient medium employed and given in Table 1 was arrived at by *a priori* reasoning. We can not say at present how many of the components of this medium are essential for growth of the parasite. Omission of the proteose peptone is, however, definitely detrimental to growth and multiplication. Recent experiments indicate that para-amino benzoic acid is probably the chief essential growth component furnished by the proteose peptone, a fact which may help to explain the observation of Coggeshall[5] that sulfanilamide will eradicate *P. knowlesi* infections in monkeys.

The techniques described here are also now being applied with the assistance of Dr. J. W. Ferrebee to the *in vitro* cultivation of human malaria parasites. Results to date have been encouraging and will be reported at a later time.

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THE ANTIBODY RESPONSE OF SWINE TO VACCINATION WITH INACTIVATED SWINE INFLUENZA VIRUS[1]

THE two human types of influenza virus, A and B, and the swine type of the agent have been obtained[2, 3, 4] in preparations of high purity by ultracentrifugation of the chorio-allantoic fluid of virus-infected chick embryos. A high degree of concentration and partial purification of the agents can be effected by sedimentation in the Sharples centrifuge,[5, 6] and procedures have been devised for practical large-scale production of virus for preparation of vaccines.[6, 7]

[5] L. T. Coggeshall, *Jour. Exp. Med.*, 71: 13, 1940.

[1] This work was supported through the Commission on Influenza and the Commission on Epidemiological Survey, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army. The work was aided also in part by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, New York.

[2] A. R. Taylor, D. G. Sharp, D. Beard, J. W. Beard (Consultant to Secretary of War and Member, Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army), J. H. Dingle and A. E. Feller, *Jour. Immunol.*, 47: 261, 1943.

[3] D. G. Sharp, A. R. Taylor, I. W. McLean, Jr., (Member, Commission on Epidemiological Survey, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army), D. Beard, J. W. Beard, A. E. Feller and J. H. Dingle, *Jour. Immunol.*, 48: 129, 1944.

[4] A. R. Taylor, D. G. Sharp, I. W. McLean, Jr., D. Beard, J. W. Beard, J. H. Dingle and A. E. Feller, *Jour. Immunol.*, 48: 361, 1944.

[5] W. M. Stanley, *Jour. Exp. Med.*, 79: 255, 1944.

[6] A. R. Taylor, D. G. Sharp, I. W. McLean, Jr., D. Beard and J. W. Beard, *Jour. Immunol.*, in press.