

It is quite possible that the α_1 -globulin, possessing the insulinlike activity, circulates in the blood stream in a complex form with another protein of higher molecular weight and isoelectric point, and that the whole complex is absorbed on the exchange resins. The treatment with 0.2M citric acid for 72 hours probably dissociates the insulin from the complex, the insulin appearing as an α_1 -globulin by paper electrophoresis. This suggestion is supported by the observation that crystalline insulin in saline or in 3-percent human albumin solution treated with cationic exchange resins retains full activity (2).

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6. Biological assays were kindly performed by Dr. K. Gunderson (University of Pennsylvania, Medical School, Philadelphia).

20 December 1957

Evidence for the Metabolism of Maleic Acid in Dogs and Human Beings

In 1951, Sacks and Jensen (1) presented evidence for the existence in maize kernels of a hydrazase, malease, for the conversion of maleic acid to malic acid. More recently, Vickery and Palmer (2) reported that the general respiration of tobacco leaves was stimulated about 60 percent when the leaves were cultured in either fumaric acid or its geometrical isomer, maleic acid. Although their data suggested that maleate behaved as an inhibitor of the activity of

the proteolytic enzymes and of the enzyme systems involved in the formation of citric acid, they noted that maleic acid entered extensively into reactions which involved decarboxylation.

We have now obtained evidence of the presence in dogs and human beings of enzyme systems capable of oxidizing maleate-2- C^{14} to $C^{14}O_2$ (3). Following the injection of 45 μ c (5.9 mg) of maleic-2- C^{14} acid into a 9.5-kg dog, arterial (femoral) blood specimens were collected in oiled, heparinized syringes at various time intervals (4). Carbon dioxide was liberated from 5 ml samples and collected as barium carbonate, and its activity was determined as previously described (5). The results (Table 1) show the increase with time of total carbon dioxide activity (per 5 ml of whole blood) as well as of "specific activity" of carbon dioxide. "Specific activity" is expressed as the percentage of injected carbon-14 per milligram of CO_2 carbon.

In Table 2 are given results that followed the intravenous injection of 90 μ c (11.8 mg) of maleic-2- C^{14} acid into a normal human subject. For purposes of comparison, data are also given for a similar experiment in which fumaric-2- C^{14} acid (90 μ c) was the injected substrate. These data show that $C^{14}O_2$ was formed readily in both cases; however, corresponding total activity and "specific activity" values were much higher in the fumarate-2- C^{14} experiment, indicating a faster reaction rate with that isomer. In the maleate experiment, the "specific activity" reached a maximum in about 70 minutes and had not declined at 90 minutes, whereas, in the fumarate experiment, the maximum was attained much sooner (in about 40 minutes) and then carbon dioxide "specific activity" declined.

As one test of the possible conversion of maleates to fumarate within the body, copper pyridyl fumarate salts (5) were made from the blood filtrates and assayed for carbon-14 activity. Whereas, in the maleic acid experiments, very little activity was found in the copper pyridyl fumarate salts (59 count/min per 5 ml of arterial blood after 23 minutes), in the fumarate-2- C^{14} experiments, considerably more activity was found (1068 count/min per 5 ml of arterial blood after 20 minutes), and this activity was evident even 70 minutes following intravenous injection into a human being (see 5, Table 2). This evidence does not rule out completely the possibility that there is a conversion of maleate to fumarate within some body tissues, with a subsequent rapid oxidation to carbon dioxide and with no entrance of fumarate into the blood stream; however, it does suggest the improbability of such a hypothesis.

Table 2. Arterial carbon dioxide activity following intravenous injection of (i) maleate-2- C^{14} or of (ii) fumarate-2- C^{14} into normal human subjects. "Specific activity" is expressed as the percentage of injected C^{14} per milligram of CO_2 carbon.

Time (min. after injection)	Total activity (count/min per 5 ml of blood)	"Specific activity"
<i>Maleate-2-C^{14}</i>		
5	159	0.000135
10	277	0.000229
20	355	0.000292
30	372	0.000309
40	410	0.000340
50	420	0.000352
70	502	0.000409
90	502	0.000410
<i>Fumarate-2-C^{14}</i>		
4	818	0.000482
14	1524	0.000910
20	1610	0.000958
40	1990	0.001210
50	1990	0.001215
70	1582	0.000914
85	1440	0.000846
96	1437	0.000846

Although the intermediate steps have yet to be elucidated, the evidence presented indicates that mammalian tissues contain enzyme systems capable of catabolizing maleic acid to carbon dioxide.

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17 October 1957

Neurotoxic Interference in *Macacus rhesus* Infected Intramuscularly with Poliovirus

Numerous experiments in *Macacus rhesus* infected with either Brunhilde (type I) or Lansing (type II) poliovirus and injected, post-infection, with certain neurotoxoids, suggest that detoxified zootoxins interfere with the experimental infection. In the past the experimental disease was induced by direct inocula-

Table 1. Arterial carbon dioxide activity following intravenous injection of maleate-2- C^{14} into a dog. "Specific activity" is expressed as the percentage of injected C^{14} per milligram of CO_2 carbon.

Time (min. after injection)	Total activity (count/min per 5 ml of blood)	"Specific activity"
5	781	0.000985
10	1042	0.001310
15	1270	0.001604
20	1373	0.001733
25	1525	0.001932
30	1632	0.002066

tion of highly paralytogenic poliovirus into the thalamus, or into the parietal or frontal lobes of the brain of the monkey. The toxoid treatment was initiated subcutaneously 1 to 7 days post-infection. The toxoids were derived from venoms of *Naja flava* (Cape cobra), *Naja naja* (Indian cobra), *Naja haje* (Egyptian cobra), and—most important—from *Crotalus terrificus terrificus* (South American rattlesnake). Toxoid production methods and interference results have been published (1, 2).

The present report is concerned with toxoid interference in *Macacus rhesus* 24 hours after intramuscular injection of a highly paralytogenic Mahoney (type I) virus. All *rhesus* used were subjected to a conditioning period of at least 10 days, at the end of which time blood cell counts were made. The presence of "naturally" occurring neutralizing antibodies against homotype virus was studied prior to the interference experiments.

As a source for inducing a uniform infection, pools of Mahoney virus obtained from monkey kidney cell cultures were used throughout these experiments, and virus potencies (TCID₅₀) were determined by titrations in the same type of culture. The infecting dose varied from 500,000 to 5,000,000 TCID₅₀ in 0.5 ml—the volume injected. With two exceptions, the virus was administered in the right deltoid muscle.

In addition to our routine *Crotalus terrificus terrificus* and *Naja flava* toxoids, a number of substances were tested as interfering agents against 3,125,000 and 5,000,000 TCID₅₀ of Mahoney virus. Two toxic substances, crotoxin (3) and a sodium salt of crotoxin, as well as two substances detoxified by acetylation, acetyl-crotoxin and acetylated *Crotalus* venom, were tested. A total of 117 animals were used in these experiments.

Group differences between treated animals and controls, within individual experiments, ranged up to 33 percent in favor of treated animals. Similar trends for entire experiments approximated 20 percent. The number of tests for each preparation was small, and no conclusion has been drawn.

Preliminary studies (2) with *Crotalus terrificus terrificus* toxoid showed it to be a promising interfering agent, and it was therefore incorporated into the standard *Naja flava* preparation in the proportion of 1 to 5. Observations made in 82 *rhesus* in three experiments have shown this combination to be the most effective toxoid to date.

Twenty-four hours after intramuscular infection with 500,000 TCID₅₀ of Mahoney virus, the mixture of *Crotalus terrificus terrificus* and *Naja flava* toxoids was administered subcutaneously, and this administration was continued on the

Table 1. Toxoid interference in *Macacus rhesus* 24 hours after intramuscular injection with 500,000 TCID₅₀ of Mahoney poliovirus (statistical analysis).

Toxoid injected (ml)	Total No. of <i>rhesus</i>	Paralyzed		Paralysis-free		Difference between test and control animals (%)
		No.	%	No.	%	
0.2	8	6	75.0	2	25.0	10.7
0.4 and 0.6	28	15	53.6	13	46.4	32.1
0.9 and 1.0	20	9	45.0	11	55.0	40.7
1.3	12	9	75.0	3	25.0	10.7
None (control saline)	14*	12	85.7	2	14.3	
All toxoid treated	68	39	57.4	29	42.6	31.8†
All comparable controls	37*	33	89.2	4	10.8	

* Twenty-three additional controls tested for *rhesus* reactivity to 500,000 TCID₅₀ of virus injected intramuscularly under identical experimental conditions were included by the statistician.

† $X^2 = 9.84$; $p = 0.01$ for $X^2 = 6.635$; $p = 0.001$ for $X^2 = 10.827$; results are significant near 0.1 percent level (0.001).

2nd, 3rd, 4th, and 7th days. Sixty-eight monkeys received treatment with varying volumes of three different lots of *Crotalus terrificus terrificus* toxoid, diluted (1:5) with *Naja flava* toxoid (Table 1). Thirty days after injection of virus the experiment was concluded, and the percentage of paralysis-free survivors in the treated groups was statistically evaluated in relation to control groups (4).

When the survivors of the interference groups receiving 0.4 ml and 0.6 ml of toxoid were combined and compared with their controls, a difference of 32.1 percent of paralysis-free animals in favor of the treated groups was observed (Table 1). This difference is statistically significant ($p < 0.05$). The 95-percent confidence interval is 5.5 to 58.7 percent.

The difference in favor of groups receiving 0.9 ml and 1.0 ml of toxoid was 40.7 percent (Table 1). The 95-percent confidence interval is 11.6 to 69.8 percent. When all toxoid treated animals were compared with all controls, and the results were statistically analyzed, the X^2 value found was 9.84. The result in favor of toxoid treatment is significant almost at the 1:1000 level (Table 1).

The degree of virus spread 24 hours after infection—that is, when treatment was initiated—was investigated. The infecting agent was found at the site of inoculation and in the corresponding brachial plexus. No virus was detected in the central nervous system. In one instance virus was found in the corresponding axillary lymph nodes and in mesenteric lymph nodes. These findings are essentially in agreement with similar experiments in cynomolgus monkeys (5).

In the screening tests for naturally occurring antibodies which had been carried out prior to selection of animals for experiments, an incidence of 4 percent

of homotype antibodies at serum dilution levels up to 1 to 10 had been demonstrated. This "natural" immunity was not sufficient to protect the monkeys against the experimental infection here described. Nevertheless, no animal with antibodies received toxoid in any interference experiment. As an extension of the antibody study, serological tests were made on 15 survivors, free of paralysis, from one experiment. While three of the six paralysis-free animals from the groups that had received 0.2 ml and 0.4 ml of toxoid, respectively, had antibodies at the 1:500 serum dilution level, only one of eight paralysis-free survivors that had received 0.6 ml and 1.0 ml of toxoid had antibodies at the same level. One paralysis-free control had antibodies at the 1:10 level. Thirty-eight days after the antibody study these animals were reinfected with 10 times the amount of Mahoney virus they had previously received. None of these animals developed paralysis. This resistance could not be correlated with the number of circulating antibodies.

Neuropathologic studies were carried out on representative paralyzed and paralysis-free animals from different experiments. The classical tissue changes in the central nervous system associated with paralysis were demonstrated in affected animals. No pathology was found in paralysis-free survivors, regardless of whether they were control or treated animals.

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23 October 1957

Shift of Oxidases with Morphogenesis in the Slime Mold, *Physarum polycephalum*

The slime mold, *Physarum polycephalum*, is one of those unusual organisms which can pass from an acellular, plasmodial stage to a cellular, spore stage during its life cycle (1), and as such represents an ultimate in morphogenetic expression. The sequential nuclear events accompanying this change in morphology include synchronous, mitotic, nuclear divisions (2) and probably meiosis when uninucleate spores are formed in the mature sporangium (3). Since both stages can be obtained at will in the laboratory, this organism lends itself well to a comparative metabolic study capable of yielding information relating to those physiological factors which may control or trigger the process of nuclear division and concomitant spore formation. Such an introductory metabolic study is, therefore, the purpose of this report and is concerned with the comparison of the activities of two oxidases in both stages of the organism (4).

Preparation of active homogenates from the plasmodial stage of this organism has been described previously (5).

Table 1. Comparison of oxidase activities in plasmodial and spore stages in the slime mold, *Physarum polycephalum*.

Cytochrome oxidase		Ascorbic acid oxidase	
K*/mg of protein N	Spore/plasmodium	K†/mg of protein N	Spore/plasmodium
<i>Spore</i>			
0.081		0.041	
<i>Plasmodium</i>			
0.028	2.9/1	0.252	1/6.1

* K = 2.3 log [reduced cytochrome c] per second.
† K = 2.3 log [ascorbic acid] per second.

The use of sonic oscillations for the disruption of spores was found to be effective in the preparation of spore homogenates (6).

Allen and Price (7) first suspected the presence of cytochrome oxidase in *Physarum polycephalum* on the basis of inhibitor studies on whole plasmodia and a positive Nadi reaction on crushed plasmodia. We have been able to demonstrate that cytochrome oxidase is indeed present in homogenates of both plasmodial and spore stages by following the enzymic oxidation of reduced cytochrome c at 550 mμ in the Beckman spectrophotometer. Although the kinetics of mammalian cytochrome c-cytochrome oxidase reaction has been reported as obeying a combination of first-order and zero-order reactions (8), the conditions of our experiments reveal only first-order concurrence with the identical system in *Physarum*. This is in agreement with the cytochrome c-cytochrome oxidase first-order reaction rates in other preparations (9). First-order velocity constants were, therefore, calculated from the slope of the plots of 2.3 log reduced cytochrome c versus time. These constants were observed to be directly proportional to the amount of enzyme used in the reaction mixture; and when they are related to protein nitrogen content, they can be used as a true measure of cytochrome oxidase activity in each cell-free stage of the life cycle of the organism.

In an attempt to measure cytochrome oxidase activity in the plasmodial stage, Holter and Pollock (10) used the indirect manometric method of Schneider and Potter (11), which requires the use of ascorbic acid as a reductant of cytochrome c. Upon subsequent examination of this system, it was shown that the electrons from ascorbic acid not only pass through the cytochrome system but are also mediated to oxygen by an atypical ascorbic acid oxidase (5). These two systems can be separated by differential centrifugation, since the ascorbic acid oxidase is not associated with a particulate fraction and can be retained in the supernatant of a 25,000 g centrifugation; this supernatant is in turn devoid of cytochrome oxidase activity. The ascorbic acid oxidase activity is atypical, since it is resistant to the usual metallorespiratory inhibitors, forms hydrogen peroxide as an oxidation end product, and requires the presence of an unknown inherent sulfhydryl compound for which diethyldithiocarbamate or its disulfide can be substituted. Since it has been shown previously that the ascorbic acid-ascorbic acid oxidase system in *Physarum* obeys first-order reaction kinetics (5),

then first-order velocity constants can also be obtained and validated as a measure of enzyme activity, as is described above.

A comparison of the oxidase activities in both the plasmodial and spore stages, shown in Table 1, reveals a shift in oxidases as the organism undergoes spore formation. Whereas there is approximately three times as much cytochrome oxidase activity in spores as in plasmodia, there is approximately six times as much ascorbic acid oxidase activity in the plasmodia as in spores.

The physiological interpretation of this shift is obscure at present, since information regarding metabolic patterns in this organism is lacking. However, these results do indicate that the atypical ascorbic acid oxidase, heretofore not rigidly established as a terminal oxidase, is not without definite function but by virtue of this shift may play an important role in the metabolism of the organism. Further, since energy is required for the nuclear divisions and formation of spore walls, a possible explanation for the increase of cytochrome oxidase activity can be offered on an energetic basis; those phosphorylation reactions necessary for the production of high-energy phosphate compounds have been demonstrated to occur in discrete stages of the cytochrome system participating in cellular oxidations (12).

This shift in oxidases may be fundamental to all organisms but may well be masked in organisms which undergo cellular division but which are constantly in the cellular state.

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4 November 1957