host animals for 14 months, but several fat pads remained only partially filled.

It appears that the transplantation of cultured cells into cleared mammary fat pads represents a technique that allows the expression of both the normal and the abnormal differentiative potential of cultured cells. Unfortunately, there is great variability between and within cultures, although every attempt is made to maintain uniformity. Variation within a single bottle is illustrated in Fig 1*F*, where two outgrowths of different character have arisen from one stroke of the scraping instrument. At present there is no obvious correlation between observable characteristics of the cultures and the nature of the outgrowths derived from these cultures.

Possibly the occurrence of a variety of abnormal growth patterns can be explained on the basis of a selection in culture of one of several minority cell types. According to such a selection hypothesis, the abnormal cells must have existed in the normal, uncultured mammary tissues but were unable to express their potential under the usual conditions of transplantation and growth in vivo. It is also plausible to assume, however, that although many of the normal differentiated properties of the cultured cells reappear after transplantation, other properties are deleted (slow growth) or added (hyperplasia).

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10 May 1965

Photosynthetic Phosphorylation: Stimulation by Pteridines and a Comparison with Phosphodoxin

Abstract. *A number of pteridine derivatives stimulate photosynthetic phosphorylation in spinach chloroplasts. In general, tetrahydro or dihydro compounds are highly active, as is one aromatic, naturally occurring compound, biopterin. The physiological characteristics of this photosynthetic phosphorylation are the same as those described for phosphodoxin. A pteridine-containing fraction from spinach, with the same* R_F *value as phosphodoxin, is also active.*

A factor which stimulates photosynthetic phosphorylation in broken chloroplasts of spinach was isolated by Black *et al.* and named "phosphodoxin" (*1*). The published ultraviolet spectrum of this substance is similar to that of a 6-alkyl-2-amino-4-hydroxypteridine. Compounds of this type (in the reduced form) are present in relatively large amounts in blue-green algae (*2, 3*) and to a lesser extent in photosynthetic bacteria (*3*); furthermore, in the case of the blue-green alga *Anacystis nidulans*, cold shock causes the release of pteridines and the concomitant loss of photosynthetic ability and viability (*4*).

These observations led us to test a number of pteridines for their ability to stimulate photosynthetic phosphorylation in broken chloroplasts of spinach. Here we report on the activity of several of the compounds tested and describe briefly the characteristics of pteridine-stimulated photophosphorylation especially with respect to its similarity to phosphodoxin-stimulated photophosphorylation.

The first compound shown to be active was 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine (TDP), the synthetic compound used by Kaufman to replace the natural cofactor for phenylalanine hydroxylation (*5*). Activity with TDP was used as a standard in further experiments with naturally occurring compounds. Spinach chloroplasts were isolated according to the method of Arnon *et al.* (*6*) except that the preparation medium contained 0.1*M* tris buffer (*p*H, 7.6), 0.35*M* NaCl, and 0.001*M* ethylenediaminetetraacetic acid. Broken chloroplasts were prepared just before use by diluting a dense suspension of whole chloroplasts with distilled water. Photophosphorylation was measured by the method of Avron (*7*), used also by Black *et al.* (*1*).

Photophosphorylation stimulated by TDP was compared in its characteristics with that stimulated by phosphodoxin. The results are shown in Figs. 1 and 2 and Table 1. At saturating concentrations ($10^{-3}M$), TDP promotes a rate of photophosphorylation approximately equal to that observed in oxygen-requiring photophosphorylation with riboflavin-5′-phosphate ($10^{-6}M$) and half that observed with phenazine methosulfate ($10^{-5}M$).

The characteristics of TDP-stimulated photophosphorylation were at least superficially similar to those of riboflavin-5′-phosphate–stimulated photophosphorylation. Other tetrahydropteridines showing similar activity and characteristics were tetrahydrofolic acid, tetrahydrobiopterin glucoside, and 2,4-dihydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

The reported absorption spectrum of phosphodoxin resembles that of an aromatic pteridine, not a tetrahydro derivative. Some aromatic pteridines were therefore tested. Under the conditions described in Fig. 1, the rela-

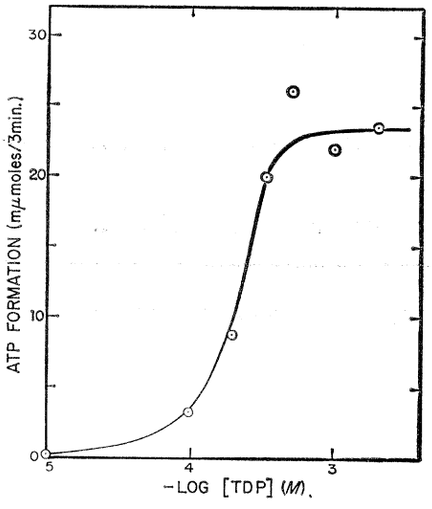

Fig. 1. Rate of formation of ATP with increasing concentrations of TDP. Reactions were carried out in 17-mm tubes containing tris buffer, *p*H 7.4 ($4.8 \times 10^{-2}M$); $MgSO_4$ ($2 \times 10^{-3}M$); inorganic phosphate, P_i ($10^{-3}M$); adenosine diphosphate ($10^{-3}M$); $^{32}P_i$ (approximately 0.5 μc); broken spinach chloroplasts (18.8 μg of chlorophyll); these were contained in a total volume of 1.0 ml. Illumination, incandescent light (14,000 lux); incubation, 3 minutes at 26°C. For other experiments mentioned in the text the TDP concentration was $10^{-3}M$.

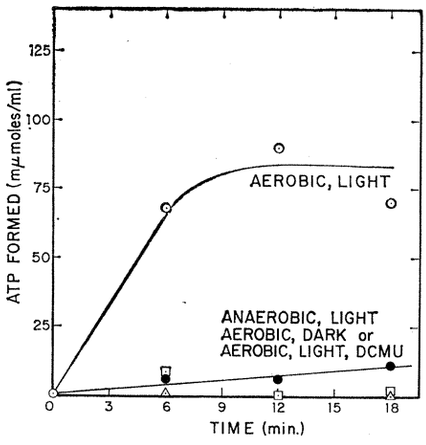

Fig. 2. Effect of light, oxygen, and DCMU on ATP formation. Components and conditions as for Fig. 1, except for chlorophyll (15.3 μg), TDP ($10^{-3}M$), and DCMU ($10^{-4}M$) when present; total volume, 2.0 ml. The reaction mixtures were incubated under aerobic conditions and light, or anaerobic conditions and light in Warburg vessels. Anaerobic vessels contained a palladium-black suspension in center well and were evacuated and filled with H_2 several times, then shaken for 8 minutes before tipping; DCMU added in ethanol (final concentration, 0.4 percent—not inhibitory to photophosphorylation).

tive rates of adenosine triphosphate (ATP) formation for the following compounds were: TDP, 100; biopterin, 86; biopterin glucoside, 13; folic acid, 12; 2,4-dihydroxypteridine, 1. [Biopterin and folic acid were added in bicarbonate buffer (*p*H 8.0; final concentration, $10^{-2}M$), which did not affect photophosphorylation.] The activity of biopterin, which also behaves similarly to TDP with regard to oxygen, light, and 3-[3,4-dichlorophenyl]-1,1-dimethylurea (DCMU), was highly significant.

The activities of some of the aromatic compounds could be increased by addition of reducing agents. As shown in Table 2, addition of TDP ($10^{-4}M$) or sodium ascorbate ($2 \times 10^{-3}M$) to the reaction mixture stimulated photophosphorylation to a greater extent than either component individually. Again, however, the case of biopterin was exceptional. Biopterin-promoted photophosphorylation was not affected by reducing agents.

The synthetic 7,8-dihydro compound, 2-amino-7,8-dihydro-6,7-dimethyl-4-hydroxypteridine [prepared as in (*8*)] was also active and, in fact, was strictly comparable to TDP with regard to concentration, dependence on light, presence of atmospheric oxygen, and DCMU inhibition. However, isosepiapterin—a naturally occurring 7,8-dihydro compound (*9*)—was not active, possibly because of its insolubility.

Finally, a pteridine-containing fraction (as determined by fluorescence, and oxidation to known products) was isolated from spinach in the following way. Spinach (about 2 kg) was boiled with 1*N* acetic acid in the presence of manganese dioxide. The filtered extract was treated with charcoal (5 g) and the charcoal was washed with water and acetone. Fluorescent material was washed from it with a mixture of ethanol and concentrated ammonia (equal volumes); this eluate was evaporated to small bulk and the concentrated solution was streaked on Whatman No. 3 MM paper (three sheets) and the chromatograms were developed with a mixture of *n*-propanol and 1 percent ammonium hydroxide (2 : 1 by volume). The material in the blue fluorescent band, R_F about 0.25, was eluted from the paper and rechromatographed in the solvent used for phosphodoxin purification (*n*-propanol, water, and 1 percent ammonium hydroxide, 3 : 1 : 2 by volume). The blue-fluorescent material, with R_F about 0.49 (the R_F of phosphodoxin in this system is 0.47), was eluted and tested under the conditions described in Fig. 1. The relative rates of ATP formation were: TDP, 100; phenazine methosulfate ($10^{-5}M$), 195; spinach fraction ($2.4 \times 10^{-3}M$ equivalents of 2,4-dihydroxypteridine as measured by fluorescence), 95. This fraction was not identical with biopterin or biopterin glucoside.

None of these pteridines was acting catalytically; the calculated ratio of pteridine present to ATP formed was never lower than 6 : 1 when the reaction was allowed to go to completion. It may be expected that the purified, naturally occurring material will display greater activity.

The general properties of pteridine-stimulated photophosphorylation suggest that these compounds may simply be acting in a redox system in somewhat the same manner as is postulated for riboflavin-5′-phosphate (*10*). The evidence would then suggest that the active redox couple would involve a dihydropteridine. Tetrahydro compounds would oxidize spontaneously to the dihydro state, and aromatic compounds would have to be reduced, presumably enzymatically, in a fast reaction in the case of phosphodoxin or biopterin, more slowly in the case of the "unnatural" compounds. In the latter case, chemical reductants might bypass this step, which might be rate-limiting, to give the higher activities observed under these conditions.

Table 1. Effect of inhibitors ($10^{-5}M$) on ATP formation. Conditions and components as for Fig. 1, except for chlorophyll (14.2 μg) and riboflavin-5′-phosphate (FMN) ($10^{-6}M$); DCMU and antimycin A were dissolved in ethanol (final concentration, 0.4 percent), and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide in KOH ($1.4 \times 10^{-4}M$ final concentration). Neither ethanol nor KOH affected phosphorylation. Data expressed as micromoles of ATP formed per milligram of chlorophyll per hour.

Inhibitor	Amount of ATP formed	
	TDP	FMN
None	24.8	30.3
DCMU	1.5	0.4
Antimycin A	13.8	15.2
2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide	12.0	9.5

The pteridines may be more intimately concerned with photosynthetic phosphorylation than is suggested above (that is, simply as redox components). The data show that relatively high concentrations of the appropriate pteridines are required to stimulate photosynthetic phosphorylation, and yet the reaction shows an oxygen dependence. The pteridine ring may, therefore, be more directly involved in phosphorylation. A plausible chemical basis for this is that a phosphopteridine (for example, a 2-amino-4-hydroxy-5,6,7,8-tetrahydropteridinyl-8-or 5-phosphonate), if oxidized, would yield

Table 2. Effect of reducing agents (TDP, $10^{-4}M$; or ascorbate, $2 \times 10^{-3}M$) on activities of aromatic pteridines. Results normalized to 100 for TDP. Reaction system as for Fig. 1, except where noted; folic acid added in bicarbonate buffer.

Pteridine ($10^{-3}M$)	Reductant	Relative rate of ATP formation
Experiment 1		
TDP	None	100
Biopterin glucoside	None	9.9
None	TDP	8.6
None	Ascorbate	11.3
Biopterin glucoside	TDP	36.6
Biopterin glucoside	Ascorbate	36.9
Experiment 2		
Folic acid	None	13.9
None	TDP	8.0
None	Ascorbate	8.0
Folic acid	TDP	44.8
Folic acid	Ascorbate	77.7

an N—P bond with a charged nitrogen, a species which would be expected to be an active phosphorylating agent. The validity of this hypothesis is being tested chemically, as well as biologically.

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27 May 1965

Dengue Type 2 Virus in Naturally Infected Aedes albopictus Mosquitoes in Singapore

Abstract. *A strain of dengue type 2 virus has been isolated from* Aedes albopictus *collected in Singapore. This is the first report of a natural isolation of dengue virus from this species, which has long been suspected as a vector in nature.*

Mosquito-borne hemorrhagic fever is a severe clinical syndrome etiologically associated with strains of dengue virus and newly recognized in southeastern Asia and India (*1–3*). During an investigation of the first recognized outbreak of the disease in Singapore in 1960–61 (*4, 5*), strains of dengue virus were isolated from the serums of two patients (*5*), five pools of *Aedes aegypti* mosquitoes, and one pool of *A. albopictus* mosquitoes. Of the two viruses isolated from patients, one was identified as type 1 dengue and the other as type 2 dengue (*5*). All of the virus isolates from mosquitoes proved to be type 2. This is the first report of an isolation of dengue virus from naturally infected *A. albopictus*, a proven efficient experimental vector of dengue (*6*) and long suspected as a vector in nature on epidemiological grounds (*7–9*).

Although *A. aegypti* has been incriminated as the principal vector of dengue for many years, it was not until 1960 that the first isolation of the virus from naturally infected mosquitoes was reported (*1*). At that time, isolations were made of dengue type 3 virus from *A. aegypti* collected in the Philippines in 1956 and of dengue type 2 from *A. aegypti* collected in Bangkok in 1958 during hemorrhagic fever epidemics. Subsequently, additional strains of virus have been isolated from *A. aegypti* collected in India (*10*), Thailand (*11*), and South Vietnam (*3*). These various isolations include all four types of dengue virus. No isolations were made from *A. albopictus*, collected and processed under similar circumstances.

In November 1960, following the peak of the epidemic in Singapore, and while cases were still occurring, a mosquito survey was initiated. Adult mosquitoes were taken routinely on a weekly basis from urban and rural houses, in diurnal and nocturnal biting collections, and from animal bait. A total of 12,505 mosquitoes, representing more than 40 species of eight genera, was collected in a 3-month period. Of these, more than 6000 female mosquitoes were processed for virus isolation.

Collected mosquitoes were held alive for a minimum of 24 hours before being processed, in order to allow for digestion of any freshly engorged blood. They were then lightly anesthetized with chloroform and accurately identified as to species. Female mosquitoes were stored at −70°C. Frozen mosquitoes were pooled by species, date collected, and location. Generally, not more than 50 mosquitoes were included in each pool. The pooled frozen mosquitoes were suspended in 3 ml of diluent (33 percent normal inactivated rabbit serum in beef heart infusion broth) and centrifuged at 10,000 rev/min for 20 minutes. A portion of the supernatant was treated with a penicillin-streptomycin mixture in the cold, and subsequently inoculated intracerebrally (0.01 ml) and intraperitoneally (0.03 ml) into two litters of 1- to 2-day-old mice. The mice were observed for signs of illness for 21 days. Brains were harvested from all sick mice, and suspended and inoculated into new groups of mice. Where no illness occurred between the 5th and 8th days after inoculation, the brains were harvested from two mice and stored.

From 4 to 5 weeks after inoculation, the surviving mice were challenged with doses of approximately 100 LD_{50} of adult-mouse–adapted dengue type 2 (Trinidad 1751) virus. Where dengue-challenge resistance occurred, the stored mouse brains were prepared as 20-percent suspensions, then diluted to 10 percent, and inoculated into mice. Portions of all mosquito suspensions and mouse brain suspensions were stored at −70°C. Part of the laboratory work was done in Singapore, where the mice were held in a mosquito-proof room. The remainder of the work was done in San Francisco, a dengue-free area.

Five strains of dengue virus were isolated from *A. aegypti* pools and one strain was isolated from a pool of *A. albopictus*. The *A. albopictus* strain (SM-18) was from a pool of 49 females that were collected in November 1960 in urban Singapore, while they were attempting to feed on human bait. The dengue virus infection rates were 0.8 per 1000 for *A. albopictus* compared to 18.6 per 1000 for *A. aegypti* (*12*).

Strain SM-18 caused illness in infant mice 10 days after inoculation with the mosquito suspension, but it adapted to mice with difficulty. Ten serial brain passages in infant mice were required before the incubation period was reduced to 7 days and a regular pattern of illness and death appeared. Of the

Table 1. Neutralization of dengue strain SM-18 virus from *Aedes albopictus* by dengue types 1, 2, 3, and 4 hyperimmune serums prepared in mice and rabbits.

Serums	Neutralizing antibody as represented by the $\log_{10}$ neutralization index with hyperimmune serums to:				
	D1 (Hawaiian)	D2 (New Guinea "C")	D3 (H-87)	D4 (H-241)	SM-18
Mouse	<1.2	2.2	<1.2	1.4	2.7
Rabbit	1.3	3.9	1.2	2.6	