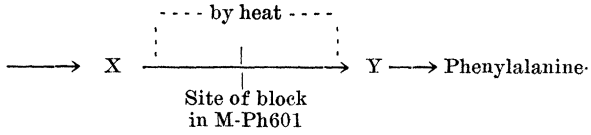
Following confirmation of the discovery that a substance that accumulates in the filtrates is responsible for the growth of the mutant, it was still uncertain whether that substance is phenylalanine or its precursor. Decisive evidence that the substance involved is actually phenylalanine was, however, obtained by paper chromatographic analysis of the filtrates together with four other unidentified amino acids (by ninhydrin test). The paper chromatogram showed the presence of phenylalanine. Furthermore, it was confirmed that M-Ph601 responds only to the eluate from the paper bearing the phenylalanine spot.

The conclusion that the phenylalanineless mutant accumulates phenylalanine itself may seem rather strange, since, according to current theory, the mutant exacting for a substance has a genetic block at a certain reaction in the biosynthesis of that substance. We could, however, find a possible explanation for this phenomenon by the following observations. M-Ph601 grown on the minimal medium-agar containing phenylalanine (10 μg/ml) was harvested and thoroughly washed with physiological saline. The washed cells were then suspended in the minimal medium (10^9 cells/ml) and shaken at 37° C. After 1 hr of shaking, the suspension was filtered with a Seitz filter. This filtrate (reinforced with the minimal medium) failed to support the growth of M-Ph601. But the same filtrate, if autoclaved (120° C, 10 min), acquired the ability to support the growth of the mutant.[2] It is obvious that this emergence of the growth-supporting activity is not due to the destruction of an inhibitor by heat, since M-Ph601 was found to be able to grow vigorously in the nonautoclaved Seitz filtrate if phenylalanine was added. Typical results of these shaking experiments are presented in Table 1.

These findings strongly suggest the following possibility. Shaking of the cells of the mutant with the minimal medium results in the accumulation of compound X, which is so unstable that it is readily convertible to compound Y, the product of the blocked reaction, by heat or other factors. Thus the filtrate, if not autoclaved, contains only compound X, substrate of the blocked reaction, and therefore is inactive. After autoclaving, however, compound Y is produced and thus the filtrate acquires the activity. Compound Y may not be phenylalanine itself but is considered to be its precursor, since paper chromatography of the autoclaved filtrate gave no spot corresponding to phenylalanine. These relations can be readily understood from the following scheme:



From these shaking experiments it may be possible to explain the results of the culture experiments as follows. In the culture experiments, too, the accumulation of compound X does take place as the first sequence. But, due to its instability, it is gradually converted to compound Y during the prolonged incubation. Phenylalanine thus produced may then be excreted into medium for reasons unknown as yet.

TABLE 1

RESPONSE OF M-PH601 TO THE SHAKING FILTRATES*

	Filtrate shaken with	Treatment	Growth
1	M-Ph601	Autoclaved	0.31
1a	M-Ph601	Not autoclaved	0.05
1b	M-Ph601	Not autoclaved plus phenylalanine†	0.14
2	Wild type	Autoclaved	0.04
2a	Wild type	Not autoclaved	0.05

* All media were used after reinforcement with 1.5 volumes of minimal medium. Growth was measured after 24 hr of cultivation and expressed in the optical density units at 460 mμ.

† Phenylalanine (3 μg/ml) is added.

[2] With the cells of M-Ph601 harvested from the peptone-bouillon media, no such phenomena were observed.

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Autocatalytic Growth of a Mutant Due to Accumulation of an Unstable Phenylalanine Precursor[1]

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Simmonds (*1*) observed that a phenylalanine auxotroph of *E. coli* accumulates a growth factor for itself. In the course of searching for intermediates between shikimic acid (*2*) and phenylalanine we encountered the same phenomenon and concluded, on the basis of paper chromatography with several solvents (using the ninhydrin reaction and bioautography for detection), that at least part of the accumulated active material is phenylalanine. This accumulation was surprising, since mutation-induced growth requirements are generally attributed to loss of activity of a single enzyme, and hence would not be expected to be accompanied by excessive synthesis and excretion of a product of the blocked reaction.

The phenylalanine accumulation became understandable when it was found (*3*) to be preceded by accumulation of an unstable substance, provisionally called prephenylalanine (PPA). The same explanation has been independently arrived at by Katagiri and Sato (*4*), whose manuscript I was privileged to

[1] Aided by a grant from the Rockefeller Foundation.

see. It therefore seemed desirable to present a preliminary report of our work at this time.

PPA has little or no growth-factor activity, either for phenylalanine auxotrophs or for mutants with earlier blocks.[2] Although PPA is stable in alkaline solution, it is very labile to acid; on incubation at 37° C it is converted within a few minutes at pH 2–3, or a few hours at 6.0–6.5, to an active growth factor. This conversion is accelerated by heat. PPA produces a bioautographic spot distinct from phenylalanine; it is ninhydrin-negative.

Because of the instability of PPA to acid, stable accumulations of it could be obtained only in media heavily buffered at pH 7.5–8.0. The usual medium, starting at pH 7.0, became acid enough during cultivation (pH *ca.* 6.5) to account for the conversion of the initially accumulated PPA into an active factor, which in turn could be converted by the cells into phenylalanine.

PPA has been partly purified by charcoal chromatography and its active product has been identified as phenylpyruvic acid. This identification is based on comparison with an authentic sample[3] with respect to the following properties: biological activity, lability to alkali, paper chromatography, $FeCl_3$ color test, ultraviolet absorption spectrum, dinitrophenylhydrazone of m.p. and mixture m.p. 161–163 uncorr. [lit. (*6*) 162–164 corr.]. Attempts to isolate PPA are in progress.

Since the reaction that is blocked in the mutant eventually takes place in the medium, an interesting autocatalytic phenomenon can be observed. When a small amount of phenylalanine is placed on the surface of solid minimal medium heavily seeded with this organism, a circle of growth of the expected diameter appears after one day of incubation. During further incubation, however, this circle spreads until it fills the Petri dish. The organisms growing at a distance from the initial stimulus are genetically unchanged; on reinoculation on minimal medium they fail to grow unless primed with a trace of phenylalanine. Furthermore, if the usual glucose in the medium is replaced by succinate the pH increases rather than decreases during growth, and the area of response to phenylalanine does not spread even though PPA can be shown to accumulate.

The accumulation of phenylalanine by phenylalanine auxotrophs now offers no obstacle to including these mutants in the large class of those that appear to lack a single enzyme.

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[2] These mutants with earlier blocks require a mixture of phenylalanine, tyrosine, tryptophan, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid (*5*). They were used in the presence of all the required factors other than phenylalanine.

[3] Kindly furnished by Dr. Alton Meister.

A Technique for Culturing *Opalina*[1]

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Efforts have been made in the past to culture the protociliates, *Opalina*[2] and *Cepedea*. Pütter (*1*), Konsuloff (*2*), Larson (*3*), Larson and Allen (*4*), and Kedrowsky (*5*) cultured opalinids in complex media with varied success. Recently, Lwoff and Valenteni (*6*) tried to culture *Cepedea* in a complex but bacteria-free medium. In some instances, they were able to continue the culture for 26 days, but they were not certain of the absence of bacteria.

The object of our experiments was to find a method by which opalinids could be maintained continuously in stock cultures. It was thought that the use of such cultures might facilitate life cycle studies and the search for a simplified medium.

A modified Boeck and Drbohlav medium (*7*) for the culture of *Entamoeba histolytica* was used. It was composed of two portions, a solid egg slant and a liquid overlay.

The solid egg slant. Two whole eggs were mixed with 25 ml of distilled water. Two-milliliter portions of this mixture were put into each of a series of test tubes (15 × 125 mm). With the tubes in a slanted position, the egg mixture was coagulated in a water bath at 80° C. *The overlay.* The solid egg slant was covered by a layer of buffered saline solution of the following composition: NaCl, 4 g; $Na_2HPO_4 \cdot 7H_2O$, 2 g; KH_2PO_4, 0.3 g; KCl, 0.2 g; $CaCl_2$, 0.02 g; $MgSO_4$, trace; $NaHCO_3$, 0.4 g; and enough distilled water to make 1 l. The overlay had a pH of approximately 7.8, apparently optimal for culturing these opalinids. Three milliliters of this solution were added to each test tube containing a coagulated egg slant, and the tubes were then plugged with cotton and autoclaved.

To get our initial inoculum, we removed the contents of the rectum of a toad, *Bufo valliceps*, and placed them in a Petri dish containing some of the buffered saline solution. This inoculum contained opalinids, bacteria, and a species of *Entamoeba*. To each culture tube, 0.2 ml inactivated human serum was added and thoroughly mixed with the liquid overlay. To each tube, bacitracin (5 γ), or penicillin G sodium (2000 units), or dihydrostreptomycin sulfate (20 mg) was also added. To each tube, 0.1 ml of the inoculum containing the opalinids and the accompanying organisms was then added and the tubes were kept at room temperature.

After 48, 72, or 96 hr, 0.2 ml of the culture from the bottom of the tube was transferred with a sterile pipet to a new culture tube, similar in all respects

[1] The authors wish to acknowledge valuable suggestions from T. T. Chen and J. L. Mohr of the Zoology Department and Miss F. Hallman of the Department of Microbiology, School of Medicine, University of Southern California.

[2] The *Opalina* used in the experiments was obtained from the rectum of *Bufo valliceps* collected in Louisiana. The species has not been identified.