EVIDENCE FOR THE DE NOVO SYNTHESIS OF THE ALPHA-AMYLASE OF *PSEUDOMONAS SACCHAROPHILA*

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ABSTRACT

EISENSTADT, JEROME M. (Brandeis University, Waltham, Mass.) AND HAROLD P. KLEIN. Evidence for the de novo synthesis of the alpha-amylase of *Pseudomonas saccharophila*. J. Bacteriol. **82**:798–807. 1961.—Chloramphenicol at a concentration of 20 μg per ml inhibited the appearance of the inducible α-amylase of *Pseudomonas saccharophila*. This inhibition was observed when induction was attempted in buffer or in a complete medium. Preinduced cells were also prevented from forming this enzyme under similar conditions. Under all the conditions tested, there was no lag in chloramphenicol inhibition, thus suggesting an absence of any protein precursor in amylase formation.

Cells suspended in a complete medium without a nitrogen source lost their capacity to form this enzyme when subsequently induced in buffer. When cells were grown in the presence of radioactive sulfate and then subjected to starvation, the radioactivity of the amino acid pool diminished only slightly. However, examination of the free amino acid pool by paper chromatography showed that the loss of enzyme inducibility was accompanied by the disappearance of glutamine, aspartic acid, and a third, unidentified, compound. Enzyme-forming ability was restored by the addition, to starved cells of casein hydrolysate, glutamate, glutamine, or aspartate. Other amino acids tested were ineffective in this regard.

When cells were induced in buffer in the presence of labeled methionine, amylase was formed at a linear rate over a 3-hr period. Furthermore, both the cellular proteins and the extracellular amylase became labeled at a linear rate.

These observations are discussed in relation to the problem of protein turnover, and are interpreted as evidence for the de novo synthesis of α-amylase in this organism.

Intrinsic to any investigation of inducible enzyme synthesis as a model for the synthesis of a specific protein is the assumption that the enzyme is synthesized de novo from low molecular weight materials.

It has been known for many years that some enzymes are formed from inactive specific precursors. Pepsinogen is converted to active pepsin by an autocatalytic reaction involving pepsin itself (Herriott, 1938); trypsinogen is converted to active trypsin in a similar manner (Kunitz and Northrop, 1936), and in rat liver, tyrosine-α-ketoglutarate transaminase is apparently formed from an inactive protein molecule (Kenny, 1960). The existence of preformed precursors of amylase has been demonstrated in cell-free pancreatic extracts (Straub and Ullman, 1955) and in cellular suspensions of *Bacillus subtilis* (Nomura et al., 1958).

Findings in other systems seem to be less suggestive of a precursor. Peters (1957) found no detectable lag in the incorporation of C^{14} from $C^{14}O_2$ into the serum albumin formed in chicken-liver slices. Studies on the origin of β-galactosidase of logarithmically-growing cells of *Escherichia coli* (Hogness, Cohn, and Monod, 1955; Rotman and Spiegelman, 1954) failed to reveal significant quantities of precursor materials within the cell. Pollock and Kramer (1958) found that no intermediate exists between free amino acids and exo-penicillinase in growing cells of *Bacillus cereus*, using the inducible strain 569, while 2% of the total enzyme formed in the constitutive strain, 569/H, could have originated from precursors.

Studies on the extracellular, inducible α-amylase (hereafter referred to as amylase) of *Pseudomonas saccharophila* revealed that virtually all of the carbon of the enzyme came from

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exogenous sources when the cells were induced in a complete medium (Markovitz and Klein, 1955*a*). By contrast, cells induced in buffer produced enzyme largely from endogenous carbon sources. Kinetic studies, performed in the presence of S^{35}-sulfate (Eisenstadt and Klein, 1959), indicated that induction in buffer yielded enzyme protein that contained only endogenous sulfur-containing amino acids. Growing cells, however, utilized endogenous sources initially, but gradually increased the rate of incorporation of externally supplied sulfur until, about 3 hr after the onset of enzyme formation, all the sulfur of the enzyme was being derived from the added sulfate. In both of these experiments, the behavior of cells induced in buffer was different than that of cells induced in complete medium, in that internal precursors seemed to be used for enzyme synthesis in preference to exogenously supplied materials.

The studies cited above did not elucidate the nature of the internal precursor materials. Nevertheless, a specific, high-molecular weight precursor is not considered likely on the following grounds: (i) Immunological studies (Sleeper and Klein, *unpublished results*) using antibody to purified enzyme failed to reveal any cross-reacting antigens in uninduced cells. (ii) Inhibitors such as dinitrophenol, azide, and arsenate were found to prevent amylase formation in buffer (Markovitz and Klein, 1955*b*). (iii) Simultaneous and sequential induction of sucrose phosphorylase and amylase in resting cells suggested a competition between these two systems for some endogenous materials (Markovitz and Klein, 1955*b*).

The experiments to be detailed here further strengthen the contention that the appearance of amylase upon induction is a consequence of the de novo synthesis of this protein.

MATERIALS AND METHODS

The organism used in these studies, *P. saccharophila*, and the conditions used in its cultivation have been described earlier (Markovitz and Klein, 1955*a*,;*b* Schiff, Eisenstadt, and Klein, 1959; Eisenstadt and Klein, 1959). The harvesting of cells, the preparation of resting cell suspensions, and the conditions of induction have been reported in these publications. Procedures for the assay of amylase, and for its purification, have also been given (Thayer, 1953; Markovitz, Klein, and Fischer, 1956).

Extraction and preparation of the free amino acid pool for chromatography. Cells were washed twice with distilled water and resuspended in water to a concentration of approximately 50 mg per ml dry weight. The suspended bacteria were heated to 100 C for 20 min to liberate the intracellular amino acids, after which the suspension was centrifuged and the clear extract carefully decanted. The pellet was washed once with 10 ml of distilled water, and the wash water was added to the original extract. The combined extract was then adsorbed (Mandelstam, 1958) to Dowex #50 resin (H^+ form) packed to a height of 5 cm in chromatographic tubes of 1 cm diameter. The columns were then washed with 50 ml of distilled water and the amino acids eluted with 25 ml of aqueous 1.5 N NH_4OH solution. The eluates were evaporated to dryness over concentrated H_2SO_4 in a vacuum desiccator. Then 2 ml of distilled water was added to the dry samples and the evaporation

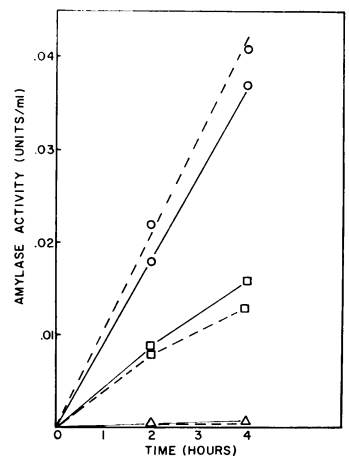

FIG. 1. *Chloramphenicol inhibition of amylase formation under growing and nongrowing conditions, using uninduced cells of Pseudomonas saccharophila. Lactate-grown cells from 250 ml of culture were harvested, washed twice with 20-ml samples of 0.033 M phosphate buffer (pH 6.8), and resuspended in 10 ml of buffer; 1-ml samples were resuspended in 50 ml of complete medium or buffer. The suspensions were then incubated for 30 min with and without chloramphenicol. At 0 time, 0.2% starch (final concentration) was added. Amylase activity determinations of the cells in complete medium (continuous lines) and of those in buffer (broken lines) were made at the intervals indicated.* ○, *controls;* □, *10 μg/ml chloramphenicol;* △ *μg/ml chloramphenicol.*

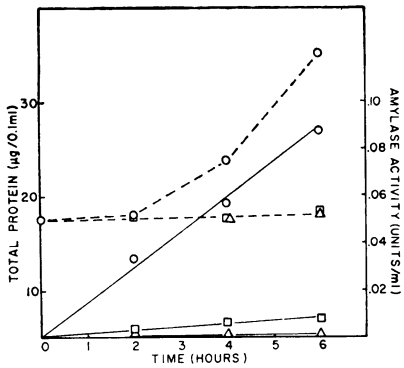

FIG. 2. *Chloramphenicol inhibition of amylase induction and cellular protein synthesis under growing conditions, using preinduced cells of Pseudomonas saccharophila. The conditions of this experiment were identical to those in Fig. 1, with the exception that starch-grown cells were used. Amylase activity determinations (continuous lines) and cellular protein analyses (broken lines) were performed at the intervals noted.* ○, *controls;* □, *10 μg/ml chloramphenicol;* △, *20 μg/ml chloramphenicol.*

repeated. The residues were transferred to chromatographic paper in a minimal amount of water.

Two-dimensional descending chromatography was used for the separation of amino acids. Prewashed Whatman no. 3mm paper, 18.5 by 22.5 in., was first run with phenol-water in the (w/v) ratio 100:39.5 (Stepka, 1952), dried, and then run with *n*-butanol-glacial acetic acid-water (100:22:50) (Stepka, 1952). After drying, the papers were sprayed with ninhydrin reagent to locate the amino acids (Woiwod, 1949).

Measurement of radioactivity. Soluble samples to be assayed for radioactivity were plated as infinitely thin preparations on ground glass planchettes. Precipitates were routinely treated by the method described by Atkinson and McFadden (1956), in which membrane filters are used. The precipitates were used in amounts small enough to obviate corrections for self-absorption. All counting was performed with an end-window, gas-flow apparatus, operating in the proportional range.

S^{35}-sulfate was obtained from the Oak Ridge National Laboratories, and S^{35}-methionine from Abbott Laboratories, North Chicago, Ill.

Protein was estimated using the method of Lowry as described by Lane (1957).

RESULTS

Chloramphenicol inhibition. Since chloramphenicol has been shown to be an inhibitor of protein synthesis (Hunter et al., 1959; Rendi, 1959) and of inducible enzyme formation (Mandelstam, 1957; Pollock and Kramer, 1958), experiments were performed to determine the effect of this compound on the ability of uninduced and preinduced (starch-grown) cells to form amylase. It was of interest to examine preinduced cells to test the possibility that a high molecular weight intermediate might be formed during amylase synthesis and accumulate in these cells. If this were true, there should be a lag before any chloramphenicol inhibition was observed.

In all cases to be described, the indicated amount of chloramphenicol was incubated with the cells for 30 min prior to the addition of inducer. Figure 1 illustrates that previously uninduced cells (lactate-grown) are inhibited approximately 60% by as little as 10 μg per ml and almost completely by 20 μg per ml. Furthermore, it is apparent that this inhibitor is effective whether induction takes place during growing or resting conditions. Similarly, in preinduced cells chloramphenicol interferes effectively with amylase synthesis as well as with general cellular protein synthesis (Fig. 2). It is interesting to note that no lag is seen in the inhibitory effect of this compound on amylase formation by preinduced cells. These cells also appear to have a greater sensitivity to chloramphenicol than do lactate-grown cells. The basis for such sensitivity has not been investigated.

Effect of nitrogen starvation on inducibility. Sucrose-grown cells were harvested, washed, and resuspended in phosphate buffer, and aliquots of this suspension were placed in each of five flasks containing sucrose and the inorganic salts normally used for growing cells, but lacking a nitrogen source. The flasks were incubated on a rotary shaker to starve the cells. At the time intervals noted in Table 1, cells were harvested, washed, and half the cells were resuspended in complete medium while the other half were suspended in buffer. Starch was then added to both cell suspensions and, after 2 hr of incubation, the cells were centrifuged and the supernatants analyzed for amylase activity. In Table 1 it is shown that starvation for as short a period as 2 hr results in a 65% inhibition of amylase

TABLE 1. *Ability of Pseudomonas saccharophila to form amylase after nitrogen starvation**

Starvation time	Medium	Amylase activity	Inhibition
min		*units/ml*	*%*
0	Complete	0.051	—
0	Buffer	0.049	—
60	Complete	0.050	—
60	Buffer	0.024	51
120	Complete	0.053	—
120	Buffer	0.017	65
180	Complete	0.047	—
180	Buffer	0.007	86
240	Complete	0.053	—
240	Buffer	0.003	94

* Sucrose-grown cells (500 ml) were harvested, washed twice with 0.033 M phosphate buffer (pH 6.8), and resuspended in 10 ml of this buffer. Aliquots of 2 ml were added to five 250-ml flasks, each containing 100 ml of starvation medium (complete medium without NH_4Cl) supplemented with 0.2% sucrose (final concentration), and incubated at 30 C. At the intervals noted, the cells were harvested and washed twice in 0.033 M phosphate buffer (pH 6.8). Half were suspended in 50 ml of complete medium, and the other half in 50 ml of buffer; 0.2% (final concentration) of starch was added to each and the flasks were incubated for 2 hr at 30 C. The cells were then harvested, and the supernatant fluid was assayed for amylase.

production in resting cells, while not affecting synthesis in complete medium. Routinely, later experiments were performed in which cells were starved for a period of 4 hr.

Effect of nitrogen-starvation on the amino acid pool. To determine what gross changes in the amino acid pool could be observed during starvation, cells first were grown on sucrose in the presence of S^{35}-labeled sulfate, and then suspended in the starvation medium for 4 hr. During this period of starvation, aliquots were removed for turbidity measurements, protein determinations, and measurements of the radioactivity of the free amino acid pool (Table 2). After the period of starvation, the cells were washed in buffer, and aliquots were resuspended in complete medium or buffer in the presence of added starch. The amylase then was isolated and assayed for enzymatic activity and radioactivity (Table 3). The results of this experiment indicate that, during starvation, cellular turbidity increases, presumably due to carbon assimilation, while no increase in protein occurs. During this period, it should also be noted that the total radioactivity of the free amino acid pool remains constant. The data of Table 3 show that these nitrogen-starved cells are capable of forming amylase only in the presence of complete medium, despite the fact that, judging from the radioactivity of the pool, the pool is far from depleted.

TABLE 2. *Changes in turbidity, free amino acid pool, and cellular protein during nitrogen starvation**

Starvation period	Culture turbidity†	Protein	Radioactivity of the free amino acid pool‡
hr		*μg/ 0.1 ml*	*count/min/0.1 ml*
0	335	31.0	7300
1	365	32.0	7250
2	392	29.0	6300
4	425	30.0	6750

* A total of 250 ml of cells, grown on sucrose in the presence of 1×10^9 count/min S^{35}-labeled sulfate, were harvested, washed twice with 0.033 M phosphate buffer (pH 6.8), and resuspended in 500 ml of starvation medium. At the intervals noted, samples were removed for protein assays, culture turbidity readings and amino acid determinations.

† Culture turbidity determinations were obtained by use of the Klett-Summerson Colorimeter (540 mμ) with distilled water as the blank.

‡ The radioactivity present in the free amino acid pool was determined in the following manner: Cells from 50 ml of culture were suspended in 10 ml of H_2O and treated as described in the methods section. Aliquots from this were measured for radioactivity. Total volume of this fraction was 1.0 ml.

TABLE 3. *Induction of amylase after nitrogen starvation**

Time	Medium	Protein	Amylase activity	Total radioactivity in amylase
min		*μg/0.1 ml*	*units/ml*	*count/min*
0	Complete	12.0	0.009	—
240	Complete	15.5	0.080	3400
0	Buffer	11.7	0.009	—
240	Buffer	11.3	0.009	0

* Cells remaining after the 4-hr period of nitrogen starvation from the experiment cited in Table 2 were washed in 0.033 M phosphate buffer (pH 6.8), and samples were resuspended under the above conditions; 0.2% (final concentration) starch was added. The cultures were incubated at 30 C, and, at the intervals indicated, samples were removed for protein and amylase determinations.

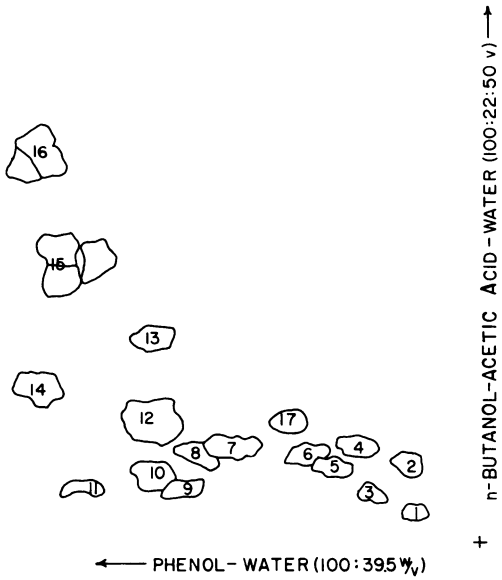

FIG. 3. *Paper chromatogram of the free amino acid pool of Pseudomonas saccharophila. Key to spots: 1, cysteic acid; 2, aspartic acid; 3, lysine; 4, glutamic acid; 5, serine; 6, glycine; 7, threonine; 8, homoserine; 9, arginine; 10, glutamine; 11, histidine; 12, alanine; 13, tyrosine; 14, proline; 15, valine, methionine, tryptophan; 16, leucine, isoleucine, phenylalanine; 17, dihydrophenylalanine or α-amino adipic acid.*

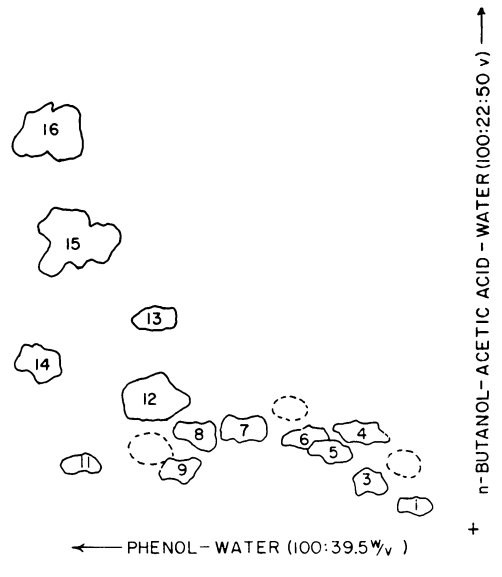

FIG. 4. *Paper chromatogram of the free amino acid pool of Pseudomonas saccharophila after nitrogen starvation. Identity of spots as in Fig. 3.*

TABLE 4. *Effect of various additions on amylase synthesis in nitrogen-starved suspensions of Pseudomonas saccharophila in buffer**

Induction medium	Additions	Amylase activity
		units/ml
Buffer	None	0.005
Buffer	0.1% Casein hydrolysate	0.050
Buffer	0.02% L-Glutamic acid	0.080
Buffer	0.02% L-Glutamine	0.050
Buffer	0.02% L-Aspartic acid	0.095
Buffer	0.02% Other amino acids†	0.005
Buffer	NH_4Cl	0.025
Complete	None	0.080
Nonstarved		
Buffer	None	0.062
Complete	None	0.076

* Conditions of starvation: complete growth medium (without nitrogen source) plus 0.2% lactate. The cells were incubated for 4 hr, washed twice with 0.033 M phosphate buffer (pH 6.8), and resuspended with 0.2% starch under the conditions as described above. They were incubated with shaking for 4 hr, after which the supernatants were assayed for amylase activity.

† Amino acids tried with no detectable effect were: DL-threonine, DL-serine, L-lysine, L-leucine, DL-isoleucine, DL-asparagine, DL-phenylalanine DL-valine, and DL-tryptophan.

The data suggest that while sulfur-containing amino acids are not depleted by nitrogen starvation, other amino acids may fall to levels so low as to prevent amylase formation before any decrease in radioactive sulfur is observed. To test this, the free amino acid pool was examined by means of paper chromatography both before and after starvation. The results are shown in Fig. 3 and 4. Of interest is the fact that the chromatogram of the pool after starvation reveals that three ninhydrin-positive spots have disappeared. These spots correspond to aspartic acid, glutamine, and one unknown spot which seems to migrate to the area normally occupied by dihydrophenyl alanine or α-amino adipic acid (Stepka, 1952).

Addition of nitrogen sources to starved cells. The effects of various amino acids on the capacity of starved cells to form amylase under resting conditions are shown in Table 4. It is clear that certain amino acids are able to restore the ability of such cells to form enzyme in buffer. The results of the examination of the free amino

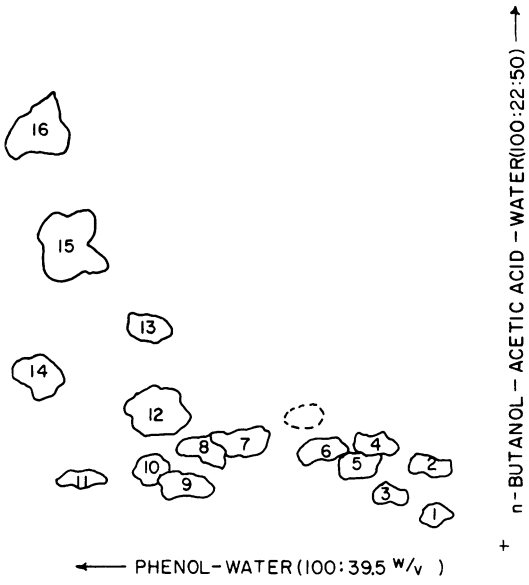

FIG. 5. *Free amino acid pool of nitrogen-starved cells induced to form amylase in buffer in the presence of* L*-aspartate. Identity of spots as in Fig. 3.*

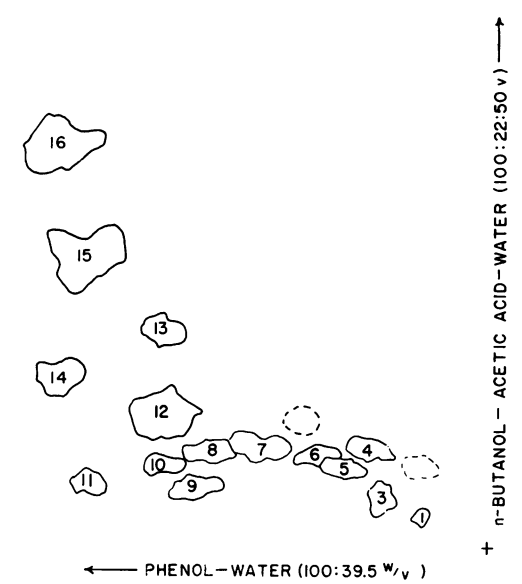

FIG. 6. *Free amino acid pool of nitrogen-starved cells induced to form amylase in buffer in the presence of* L*-glutamine. Identity of spots as in Fig. 3.*

acid pools of these cells are shown in Fig. 5 and 6, from which it is seen that incubation with several additives has resulted in a restoration of the corresponding missing amino acids of the pool. Incubation with aspartate results in the appearance of both aspartic acid and glutamine among the acids of the pool, while glutamine addition yields only this compound in the pool. Thus, these results suggest that amylase can be formed in the absence of aspartate and the third compound. One cannot conclude from these results that aspartic acid or the third acid are not incorporated into amylase protein. It is possible, for example, that incubation of starved cells with glutamine yields aspartate in amounts too small to be detected on chromatograms, particularly after 4 hr of amylase synthesis. It is also interesting to note (Table 4 and Fig. 4) that cells induced in buffer after starvation with no additives show no replenishment of the amino acids of the pool and no amylase formation.

Preincubation of starved cells with amino acids. It was of interest to determine whether the amino acids which could reverse the nitrogen starvation effect had to be present during induction. Therefore, sucrose-grown cells were starved for 4 hr, and aliquots were resuspended in phosphate buffer containing only the various amino acid additives to be tested. After 60 min incubation, the cells were washed and induced

TABLE 5. *Effect of preincubation with certain compounds on amylase formation by nitrogen-starved cells of Pseudomonas saccharophila**

Preincubation medium	Additions	Amylase activity
		units/ml
Buffer	None	0.003
Buffer	0.1% Casein hydrolysate	0.059
Buffer	300 μg/ml Glutamic acid	0.041
Buffer	300 μg/ml Aspartic acid	0.046
Complete	None	0.050

* Sucrose-grown cells (250 ml) were harvested, washed twice with 0.033 M phosphate buffer (pH 6.8), and resuspended in 500 ml of starvation medium supplemented with 0.2% sucrose. After 4 hr incubation, the cells were harvested, washed twice with buffer, and samples were suspended in buffer with the supplements noted. Each sample was incubated for 60 min at 30 C with shaking, after which the samples were harvested, again washed with buffer, and resuspended in 50 ml of buffer to which was added 0.2% starch. Induction was continued for 4 hr, after which the supernatants were assayed for amylase activity.

TABLE 6. *Sucrose-grown cells induced in buffer in the presence of S^{35}-labeled methionine**

Time	Total cellular protein	Amylase	Radioactivity	
			Cell protein	Amylase
min	*μg/0.1 ml*	*units/ml*	*count/min/ 2 ml*	*count/min/ 22 ml*
0	24.3	0.004	520	—
20	—	0.007	1160	115
40	—	0.013	1800	275
60	22.8	0.017	2780	420
120	24.5	0.035	4010	850
180	24.0	0.054	5700	1300

* Sucrose-grown cells (500 ml) were harvested, washed twice with 0.033 M phosphate buffer (pH 6.8), and resuspended in buffer. Aliquots of this suspension were added to 173 ml of buffer to give a Klett-Summerson reading (540 mμ) of 150. Cells were incubated at 30 C, with shaking for 15 min, after which 2 ml of buffer containing S^{35}-methionine (9×10^7 count/min) and 0.2% starch was added and the incubation continued. At the intervals noted, samples were removed for protein and amylase analyses and for radioactivity determinations. See text for further details.

in buffer. Table 5 illustrates that after starvation, the depleted amino acids may be restored to the pool prior to induction. It should be noted in this connection that a greater concentration of the amino acid supplement is needed under these conditions than was necessary under conditions used in the experiment shown in Table 4.

S^{35}-labeled methionine experiments. If a radioactive amino acid, which is a constituent of amylase, could be introduced into the amino acid pool, kinetic experiments on its incorporation into enzyme protein could be performed (see also Pollock and Kramer, 1958). L-Methionine was chosen for this purpose, since it appears that all the sulfur of amylase is present in this amino acid (Eisenstadt and Klein, 1959).

The technique used to obtain fully labeled amino acid pools is based on the observations of Mandelstam (1958), who found that incubation of *E. coli* with a labeled amino acid resulted in immediate equilibration of the internal amino acid pool with the extracellular amino acid that was supplied. This method makes it possible to obtain cells with unlabeled cellular proteins but with fully labeled amino acid in the pool. Under these conditions, cells of *P. saccharophila* induced in buffer should produce labeled amylase with no lag in specific radioactivity, provided that the cells do not contain significant quantities of a preformed precursor.

Cells for this experiment were grown in sucrose, washed, and induced in the presence of S^{35}-labeled methionine. Immediately after addition of the methionine, and also at the intervals noted (Table 6), 25-ml samples were removed, chilled to 0 C, and centrifuged. The supernatant fluids from the centrifuged cells were retained and analyzed for amylase activity. The amylase was then purified and assayed for radioactivity. Also at the times noted, 2-ml samples were added to 2 ml of 10% trichloroacetic acid (TCA) containing 600 μg per ml of unlabeled methionine plus unlabeled cells (equivalent to 1 mg of protein) as carrier. The TCA precipitates were heated for 25 min at 90 C, and transferred to membrane filters with three 5-ml washings of hot 5% TCA containing 300 μg per ml of unlabeled methionine. Preliminary experiments had determined that, under the conditions described, it was not necessary to treat the samples with organic solvents to remove lipids. The removal of all samples was accomplished in approximately 45 sec and was always performed in the same order to maintain the time intervals.

Table 6 shows that during the course of this experiment no increase in cellular protein was detected, while appreciable amounts of amylase were produced. It is clear that both the amylase and the cellular proteins contain radioactivity

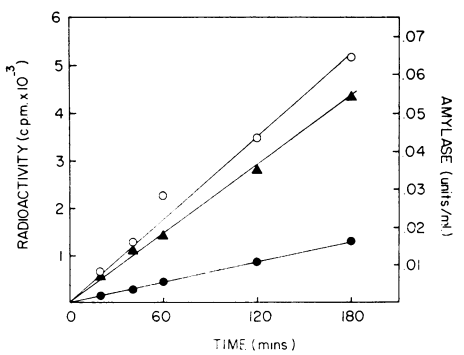

FIG. 7. *Incorporation of S^{35}-labeled methionine into amylase and cellular proteins during induction in buffer. For details see Table 6.* ○, *radioactivity per 2 ml of cellular protein;* ●, *radioactivity per 22 ml of amylase protein;* ▲, *amylase activity per ml.*

from the earliest intervals examined. The data have been plotted in Fig. 7, which illustrates that both cellular proteins and amylase protein incorporate S^{35}-labeled methionine at a linear rate, with no detectable lag. This indicates that, during the interval examined, penetration of methionine into the cell is not rate-limiting. It should also be noted that amylase activity increases at a linear rate during this period.

DISCUSSION

The chloramphenicol experiments support the proposal that amylase is formed de novo. If a constitutive precursor were present in uninduced cells, chloramphenicol would be expected to have little immediate effect upon subsequent induction of these cells. On the other hand, cells actively utilizing starch might accumulate appreciable amounts of a precursor and thus be able to form active enzyme in the presence of chloramphenicol. Since both lactate-grown (uninduced) and starch-grown (preinduced) cells were inhibited by as little as 20 μg per ml of chloramphenicol, with no observed lag, it seems unlikely that either kind of cell contains a specific enzyme precursor.

Starvation experiments with *P. saccharophila* have shown that periods of nitrogen starvation result in an inability of the cells to synthesize amylase under resting conditions. Examination of the free amino acid pool at this time shows that the total amount of sulfur-containing amino acids in the pool remains relatively constant, but chromatograms reveal that three ninhydrin-positive spots, corresponding to aspartate, glutamine, and one other unknown compound, disappear from the pool. When amino acids that have disappeared are added to nitrogen-starved cells, amylase can be induced in buffer. (In this regard, it should be noted that D-aspartate is unable to replace the L-isomer in the pool for the subsequent induction of amylase.) This reversal of nitrogen starvation is also observed when NH_4Cl or casein hydrolyzate is added, or if the induction takes place in complete medium. It is evident from these results that protein turnover, which does occur in these cells (Eisenstadt and Klein, unpublished observations), is not effective in preventing the net loss of aspartic acid, glutamine, and the third unknown compound from the pool. Of interest is the observation that, while the radioactivity of the pool remains constant during starvation of sucrose-grown cells, subsequent induction in unlabeled complete medium results in the incorporation of radioactivity into amylase protein.

While the experiments with starved cells do not prove that amylase is synthesized de novo, they do support this contention. The strict correlation between the loss of certain acids of the amino acid pool and the subsequent inability to form enzyme is most readily explained on such a basis.

It has been pointed out (Pollock and Kramer, 1958) that, if extracellular radioactive amino acids equilibrate with the intracellular amino acid pool without any appreciable lag, any delay found between the addition of these materials and their incorporation into a specific protein may be regarded as evidence for a preformed precursor of the enzyme. Furthermore, the absence of a lag in incorporation would rule out a precursor without any assumption of equilibration of the added radioactive amino acid.

In this type of experiment, using S^{35}-methionine, it was found that, during the period of induction in buffer in the presence of the radioactive amino acid, incorporation into cellular proteins occurred at a linear rate with no lag (Fig. 7). This eliminates any uncertainty concerning equilibration of the external amino acid with the internal pool. Since the rates of amylase formation and of enzyme labeling were both linear, it must be concluded that enzyme protein was being labeled at a constant rate throughout the course of this experiment. In the absence of any lag of S^{35}-methionine incorporation into amylase, it is therefore reasonable to conclude that the formation of amylase under resting conditions is accomplished de novo from low molecular weight compounds pre-existing in the cells or in the medium prior to induction. Of course, it could be argued that this conclusion is justifiable only in regard to methionine; that methionine could add on to a preformed precursor to form the active enzyme. However, this contention would appear to be unlikely in view of all the other evidence cited above, and because we have also shown that added glutamine and aspartate are incorporated into amylase protein in resting cell suspensions (Eisenstadt and Klein, 1960).

On the basis of these and earlier observations on the formation of amylase in this organism,

the following picture seems plausible. Under the conditions used in these studies, growth of the cells ceases when the level of at least one amino acid drops below a certain threshold, leaving a variety of other amino acids (including sulfur-containing acids) in the pool. These acids can be used for the synthesis of proteins, including amylase, that do not contain the depleted amino acids. With nitrogen starvation, the composition of the pool changes again as a result of the depletion of glutamine and the other two acids, and, under these more austere circumstances, amylase synthesis becomes impossible. If such starved cells are capable of synthesizing any proteins at all, these should be relatively simple in composition. The advantage, to an organism like *P. saccharophila*, of this step-wise depletion of the amino acid pool is obvious. Growth of uninduced cells would cease well before all the amino acids of the pool were incorporated into proteins, and the resultant cells would now be in a position to form amylase preferentially upon induction (Eisenstadt and Klein, 1960).

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