TYROSINE AND PHENYLALANINE AMMONIA LYASES IN SPOROBOLOMYCES ROSEUS

by

EDITH ELLEN CAMM

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Department of Botany

The University of British Columbia
Vancouver 8, Canada

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ABSTRACT

The enzymes phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) were studied in the yeast Sporobolomyces roseus (Kluyver and van Neil). Cells grown on a glucose-salts medium were ground with alumina, and the cell-free buffer extract was fractionated with ammonium sulfate. Enzyme activity was assayed by measuring spectrophotometrically the cinnamic acid and p-coumaric acid produced from phenylalanine and tyrosine respectively. Further attempts at purification resulted in the inactivation of the TAL. Although the two enzymes were not separated by the purification procedures used, there is some evidence that the deamination of phenylalanine and tyrosine are catalyzed by different proteins, and not by a single enzyme with wide specificity. TAL appears to be precipitated by lower concentrations of ammonium sulfate than is PAL. The pH curves of the two enzymes are different. The specific activities of the two enzymes can be changed relative to one another in the cell by changing the medium upon which the cells were grown. The rates of production of the two enzymes vary independently during the growth of the cells.

While the proteins are probably distinct, the production and activity of each enzyme seem to be under common control. Peak production of both enzymes occurs during late logarithmic-early stationary phase in the growth of a batch culture. Replacement media containing either phenylalanine or tyrosine stimulate the production of both PAL and TAL. Similarly, media containing cinnamic acid or p-coumaric acid repress the formation of the enzymes. Studies using labelled substrate show that both products inhibit the action of both enzymes.

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INTRODUCTION

The enzyme phenylalanine ammonia lyase (E.C. 4.3.1.5) (Koukol and Conn, 1961) catalyzes the conversion of phenylalanine to cinnamic acid and ammonia (Figure 1). Tyrosine ammonia lyase (E.C. 4.3.1.) (Neish, 1961) similarly deaminates tyrosine, with the formation of p-coumaric acid and ammonia (Figure 1).

The control of production of both PAL and TAL has been investigated in only a few cases. In sweet potato, the enzymes PAL and TAL, although separate proteins, are both produced after wounding or fungal infection. The rather general stimulus induces the formation of both enzymes (Minimikawa and Uritani, 1965a). Preparations of the inducible PAL from Rhodotorula glutinis also show TAL activity (Ogata et al, 1967b). In the first case, the stimulus to the sweet potato tissue results in the production of two proteins. In the second case, the situation is not so clear. Ogata et al (1967b) suggest that the same protein might catalyze the deamination of both phenylalanine and tyrosine. This would imply that the deamination of tyrosine is stimulated by the presence of phenylalanine.

The yeast Sporobolomyces roseus in the Sporobolomycetaceae produces both PAL and TAL when grown on medium containing malt extract (Moore et al, 1967). The following study was undertaken to investigate the nature of the enzymes, whether one protein or two, and to determine the nature of the control over their production.

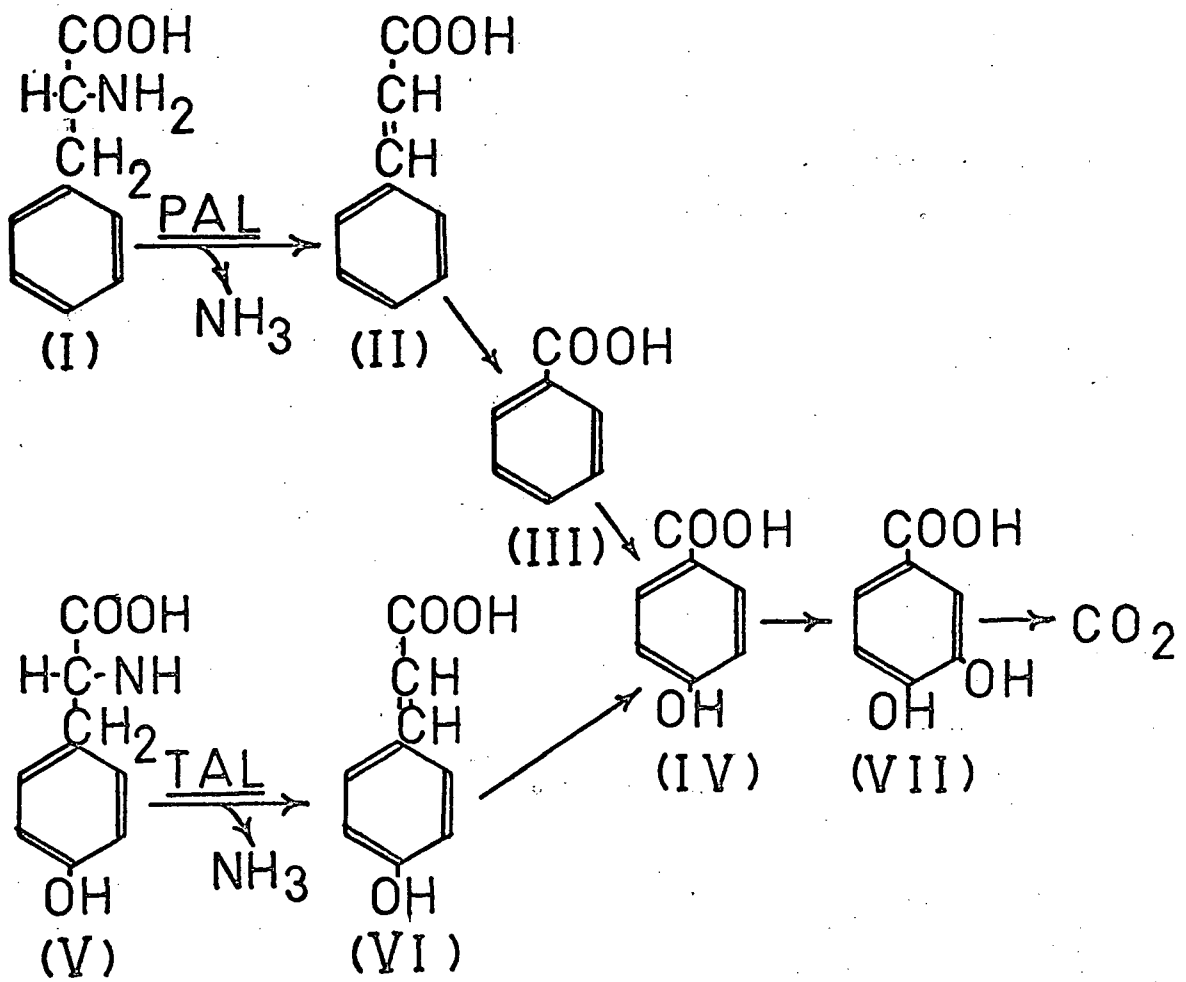


FIGURE 1

Degradation of phenylalanine and tyrosine by Sporobolomyces roseus (Moore et al, 1967)

(I) phenylalanine; (II) cinnamic acid;
(III) benzoic acid; (IV) p-hydroxybenzoic acid;
(V) tyrosine; (VI) p-coumaric acid;
(VII) protocatechuic acid

LITERATURE REVIEW

Since the discovery of phenylalanine ammonia lyase (PAL) by Koukol and Conn (1961) and of tyrosine ammonia lyase (TAL) by Neish (1961), a growing body of literature has accumulated about the two enzymes. While there have been as yet no reports of the enzymes in algae, mosses or bacteria (save for one reported occurrence in Streptomyces (pers. comm. from L. Vining to G.H.N. Towers)), both enzymes have been found together in a liverwort, several ferns, lycopods, gymnosperms, monocotyledons and dicotyledons (Young, Towers and Neish, 1966). The enzymes have also been found in many fungi, including wood-rotting basidiomycetes (Powers et al, 1965, Bandoni et al, 1967) and yeasts in the Sporobolomycetaceae (Bandoni et al, 1967) and Cryptococcaceae (Ogata et al, 1967a).

In Sporobolomyces roseus, the degradation of phenylalanine and tyrosine occurs as shown in Figure 1 (Moore et al, 1967). But in general, to explain the ammonia lyases as simply the first step in the oxidative degradation of phenylalanine and tyrosine is not very satisfactory. In the first place, there would seem to be very little substrate for the enzymes produced by species like Lentinus lepideus, which grows on wood (Power et al, 1965). Secondly, many microorganisms ordinarily convert the aromatic amino acids to homogentisic acid through the intermediary formation of phenylpyruvic acid and p-hydroxyphenylpyruvic acid (Rogoff, 1961; Evans, 1963). Since Schizophyllum commune produces cinnamic and p-coumaric acids as well as phenylpyruvic and p-hydroxyphenylpyruvic acids from the aromatic amino acids, both pathways are presumably operating in this species (Moore and Towers, 1967). Some strains of Rhodotorula produce phenylpyruvic acid from phenylalanine when grown on

certain media, although others produce cinnamic acid (Ogata *et al*, 1967a). *Tilletiopsis* sp. produces no ammonia lyases unless the organism is grown for several hours on phenylalanine (K. Moore, pers. comm.). In short, many fungi which are able to degrade the aromatic amino acids *via* the phenylpyruvic acid pathway can also produce an enzyme which catalyses the first step in a second pathway of degradation.

In some fungi, only the cinnamate pathway seems operative. In *Sporobolomyces roseus*, both PAL and TAL are quite active, and only the cinnamate pathway has been found in cells grown on malt medium (Moore *et al*, 1967). Whether this organism is capable of producing phenylpyruvic acid and the hydroxylated derivative from the aromatic acids is not known.

There is a possibility that the fungi which possess both pathways have a requirement for cinnamic acids. Hispidin, one of the few fungal compounds obviously bearing a cinnamate skeleton, has been found in the bracket fungi *Polyporus hispidus* and *P. schweinitzii* (Bu'Lock 1967, and references therein). Bu'Lock observed that the quantity of hispidin found in the sporophore decreased as the organ became more woody with the advancing season. He hypothesized that the hispidin might be polymerizing and cross-linking with the cell wall material to form supporting material in the sporophore. In general, however, the cinnamate skeleton produced by the action of the ammonia lyases from the aromatic amino acids is seldom incorporated into secondary fungal metabolites.

In contrast to the situation in the fungi, in higher plants cinnamic acid derivatives are common and important. Phenylalanine has been shown to be incorporated into lignin as cinnamic acid, while in grasses tyrosine as well as phenylalanine is incorporated into lignin, but *via* p-coumaric acid (Brown, 1961). Lignification in bamboo is correlated with the activity of PAL and TAL

(Higuchi et al, 1967). PAL development parallels lignification in buckwheat (Yoshida and Shimokoriyama, 1965). In these plants, the enzymes have been strongly implicated in lignin synthesis.

PAL seems to be involved in other processes as well as lignification. In strawberry, PAL activity increased on exposure of leaf discs to light (Creasy, 1968). At the same time, the quantity of flavonoids increased. In this plant, red and far-red light increase flavonoid production in the leaf discs, but do not affect enzyme activity. On the other hand, phytochrome has been implicated in PAL regulation in Sinapis alba, since PAL activity is increased by far-red light (Durst and Mohr, 1967). Blue light is effective in PAL induction in gherkin (Engelsma, 1967). In Fagopyrum esculentum, phytochrome has been shown to control both anthocyanin and PAL production (Scherf and Zenk, 1967).

The changes in metabolism in injured tissue seem connected with the ammonia lyases. In sliced potato tubers, the buildup of chlorogenic acid is very closely connected with the light-induced induction of formation of PAL protein (Zucker, 1965, 1966, 1968). A similar phenomenon has been observed in sweet potatoes which have been infected with black rot (Minimikawa and Uritani, 1965a). After wounding or infection of the tissue, the PAL activity rose to levels 150 times normal. The levels of TAL rose also, but not to such high levels. The change of activity in both enzymes showed a correlation with the pattern of accumulation of polyphenols in the tissue. The activity of the enzymes would provide the phenylpropanoid skeleton for the synthesis of the polyphenols.

While there are reports that PAL occurs alone in some plants (Young and Neish, 1966), TAL occurrence may be more widespread than first thought. Work on partially purified preparations of TAL suggest that it is a very labile

enzyme (Koukol and Conn, 1961), and loss of TAL activity might occur under assay conditions which do not affect PAL activity. Then, too, the plant might lose TAL activity as it ages. Neish (1961) found no TAL in 30- to 40-day old alfalfa plants, although later work (Young and Neish, 1966) showed that young plants did contain the enzyme. In bamboo, the zone of enzyme activity in the stem moves upward as the stem elongates; older tissue has low activity (Higuchi, 1966).

A third possible cause of low values lies in endogenous inhibition of the enzyme by material in the cells. Chrysanthemum shows no PAL or TAL activity, and acetone powders of this plant contain a potent inhibitor of the activity of preparations from other sources (Young and Neish, 1966). Such endogenous inhibitors may account for some negative tests of activity which have been reported.

In view of the many reported occurrences of the enzymes, we may assume that PAL, at least, is very important and almost ubiquitous in higher plants. These enzymes seem to be the link between the aromatic amino acid metabolism and much of the secondary biosynthetic activity of the plant.

The situation in the fungi regarding these enzymes is quite different. Many taxonomic groups (including the whole of the class Ascomycetes) have not been screened. Even in the Basidiomycetes, the distribution of the enzymes is not general, for they appear to be lacking in some genera such as Poria and Polyporus (Bandoni et al, 1967). As in the higher plants, PAL is the more common enzyme, although TAL alone has been reported in the uredospores of Puccinia graminis (pers. comm. by A. Jackson to G.H.N. Towers). Although there is no information on the presence of endogenous inhibitors, like that found in Chrysanthemum, some negative reports might well be due to inhibition of the enzymes which were active in vivo.

As in the higher plants, enzyme production is correlated with a certain stage of growth. In Rhodotorula, the peak of activity occurred just as the batch culture entered the stationary phase of growth (Ogata et al, 1967a). Slow growing fungi might have already stopped producing the enzyme before they were assayed.

In some fungi, the enzymes are inducible. Except under certain growth conditions, assays would yield negative results. In Tilletiopsis, phenylalanine seems to induce the formation of PAL (K. Moore, pers. comm.). The same phenomenon has been noted in Rhodotorula glutinis (Ogata et al, 1967a). The production of the enzyme PAL seems to be under strict control.

MATERIALS AND METHODS

Reagents

Radioactive phenylalanine and tyrosine were obtained from New England Nuclear Corporation. Cinnamic acid 3-^{14}C was obtained from Merck, Sharp and Dohme Limited. All non-radioactive compounds were reagent grade and were obtained from several commercial sources.

Culture of the organism

The organism, Sporobolomyces roseus Kluyver and van Neil (UBC 901), was kindly supplied by Dr. R. Bandoni. Cells were grown in flasks in shake culture at 25°C in a New Brunswick Psychrotherm shaking incubator. Into each one-litre flask were dispensed 250 ml of minimal medium (Vogel's salts, 1% glucose (Vogel, 1956)).

Compounds other than glucose were also used as the sole carbon source. Cells were inoculated into Vogel's salts containing 0.01% of the compound acting as carbon source. Two more transfers of cells into fresh media were carried out to ensure that any observed growth of the cells was due to the carbon source supplied. Because of its low solubility, cinnamic acid was supplied as the sodium salt. The acidity of the medium between pH 5.5 and pH 7.5 seemed to make little difference to the growth of the cells.

Growth was followed by changes in absorbance as measured at 600nm in a Bausch and Lomb Spectronic 20 colorimeter. Cell counts were made with a hemacytometer. Since absorbance appeared to vary directly as cell number (with cells from cultures of less than four days' growth), the simpler measurement

of absorbance was routinely used. After three to four days' growth, however, often chains of cells appeared. These and pseudomycelia made cell counts or optical density measurements unsatisfactory.

Cells were harvested by centrifugation, washed twice with distilled water, and stored frozen at -20°.

Enzyme assay

The assay procedure was similar to that used by Subba Rao et al (1967) for Ustilago PAL. Frozen cells were ground by hand with one to two times their weight of powdered alumina in a chilled porcelain mortar for fifteen minutes. The suspension was extracted with two times the volume of .05M tris buffer, pH 8.0, [illegible] as weight of cells used. This slurry was centrifuged at 0° to 4°C for twenty minutes at 4100x g in a Sorvall RC2B refrigerated centrifuge. The turbid pink supernatant was used as the crude enzyme.

To assay TAL, 5 µmoles of L-tyrosine was added to 0.5 ml of the above crude extract, and the volume made up to 3.0 ml with .05 M tris buffer at pH 8.8. The mixture was incubated at 37°C for sixty minutes. The control was identical except for the addition of 1.0 ml of 1.0 ml of 1N HCl. At the end of the incubation period, the reaction was stopped with the addition of 1.0 ml of 1N HCl. The reaction mixture was extracted with 5 ml of peroxide-free ether and an aliquot of this evaporated to dryness. The aliquot was dissolved in 0.05N NaOH and its absorbance measured at 333 nm measured with a Unicam recording spectrophotometer. The difference between this absorbance and that of the extracted control was converted to µg of p-coumaric acid.

To assay the PAL content of an extract, the same procedure was followed as with the TAL assay, except that 5 µmoles of L-phenylalanine were used as substrate, and absorbance was measured at 268 nm.

Activity was expressed in units; one unit of TAL is the amount of enzyme which will catalyze the conversion of 0.01 μmoles of tyrosine to p-coumaric acid in one hour at 37°C. Similarly, one unit of PAL catalyzes the conversion of 0.01 μmoles of phenylalanine to cinnamic acid.

Preparation of acetone powder

Stored frozen cells were thawed, then held at 0°C for the duration of the extraction. The thawed cells were suspended in -20°C acetone, filtered on a Büchner funnel, washed with more acetone and dried on the funnel. The powder was suspended in cold .05M tris buffer pH 8.8, and the filter paper from the funnel rinsed with more buffer. The suspension and rinsings were made up to 5.0 ml, and were gently mixed to achieve a homogeneous suspension. An aliquot of this was used in place of the crude extract in the standard enzyme assay. Cetyl trimethyl ammonium chloride was added to the reaction mixture at the rate of approximately 0.1 mg per 20 ml acetone powder.

Protein measurement

The method of Lowry et al (1951) was used for the measurement of protein in the crude fraction. Spectrophotometric methods could only be used with fractions from which the nucleic acids had been removed; otherwise the 260 nm absorption was too high.

Ammonia determination

A simple assay was set up to determine the ammonia content of the growth medium. To 2.0 ml of a 1/100 dilution of the supernatant from a cen-

trifuged aliquot of a growing culture was added 0.05 ml of Nessler's reagent (British Drug House). The solution was immediately mixed. No more than five minutes later, the optical density of the solution was measured at 500 nm on a Spectronic 20 colorimeter. The reading was converted to μmoles of ammonia per ml.

Glucose determinations

The glucose content of the centrifuged growth medium was measured semi-quantitatively with commercial glucose-oxidase paper (Lilly 'Tes-Tape').

Identification of the products of the reactions

Cells of known high TAL activity were ground and extracted in the usual manner. Crude extract (4.0 ml) was allowed to react with 8.0 μC (3.2 μmole) of tyrosine 3-^{14}C for one hour at 40°C in the presence of 10 ml of .05M tris buffer, pH 8.8. At the end of one hour, the reaction mixture was acidified and continuously extracted for four hours with ether. The extract was evaporated to dryness, then taken up in ethanol and spotted on Whatman #1 chromatography paper. The chromatograms were run two dimensionally in benzene:acetic acid:water (10:7:3, upper phase) and then 2% formic acid. Radioautographs were prepared with Kodak Medical X-ray film (estar base). The Rf's of the spots on the film were compared with the Rf's of authentic *cis* and *trans* *p*-coumaric acid and *p*-hydroxyphenylpyruvic acid. This latter has the same spectrum as *p*-coumaric acid, and could not be distinguished from it by spectrophotometric means.

The UV spectrum of the reaction product was examined. A reaction was set up using 5 μmoles of non-radioactive L-tyrosine. Extracts of the mixture

were chromatographed as above. The areas on the chromatograms corresponding to the darkened areas on the radioautographs described above were eluted with 80% alcohol. The spectra of the eluted compounds were compared to those of authentic p-coumaric acid.

A similar procedure was followed to determine the products of the PAL reaction. Crude extract (1.0 ml) was allowed to react with 0.4 μC of phenylalanine 1-^{14}C (0.16 μmoles) and 0.5 μmoles of L-phenylalanine for one hour at 40°C in the presence of 2.0 ml of .05M tris buffer, pH 8.8. After extraction with ether, the product was chromatographed with authentic cinnamic acid 2-^{14}C as a standard in the above solvents. Radioautographs were prepared.

Studies using replacement media

Cells were grown in the normal manner in minimal medium for 48 hours. At the end of this time, they were harvested aseptically and washed twice with sterile distilled water. The cells were suspended in the same volume of fresh medium containing salts and one or more of several compounds as carbon source.

The resuspended cells were once again incubated with shaking. Aliquots were removed for analysis at various times after resuspension. The optical density of the cell suspension was measured, and an acetone powder was prepared from the washed cells.

Partial purification of the enzymes PAL and TAL

A crude extract was prepared as described earlier from cells of known high activity. The crude extract was stirred gently for fifteen minutes with a 2% solution of protamine sulfate in water, added at a rate of 3 ml per 10 ml

of crude extract. Centrifugation at 12,000x g for fifteen minutes resulted in a pale pink opalescent solution which was fractionated using ammonium sulfate. Solid ammonium sulfate was added with stirring to bring the solution to various levels of saturation. Pellets were collected by centrifugation at 20,000x g. Preliminary experiments had shown that the enzymes were quite stable in the ammonium sulfate solution, even at the lower pH's caused by the addition of the salt, so no attempt was made to keep the pH above neutrality. The drained pellet was suspended in fresh buffer, pH 8.0, in half the volume from which the pellet was collected.

The most active fraction was dialyzed overnight in the cold against 50x the volume of M/20 tris buffer pH 8.0. Since much of the TAL activity was lost overnight by this treatment, most preparations were instead passed through a column of Sephadex G-25 at 6-8°C to remove the ammonium sulfate. This partially purified fraction was stored at -20°C until needed. Storage of much more than a week resulted in some inactivation of TAL.

Adsorption of inactive protein on Alumina C γ gel was attempted. The effluent from a G-25 column (10 ml at 10 mg protein per ml) was adsorbed on 40 mg of gel in 0.01M phosphate buffer, pH 7. Elution of the enzyme from the gel was not successful. Even 0.1M buffer at pH 8 resulted in considerable loss of enzyme. Since the TAL was present in quantities barely measurable, this procedure was discontinued.

Fractionation on Sephadex G-200 was attempted, but again, the TAL function was either inactivated or diluted below the limit of resolution of the assay system.

RESULTS AND DISCUSSION

Identification of the products of the reactions

The major product of the Sporobolomyces roseus TAL reaction was similar to trans-p-coumaric acid with respect to Rf in two solvent systems, colour reaction with diazotized p-nitroaniline (Ibrahim and Towers, 1960), and fluorescence in ammonia vapour (Table I). A minor product of the reaction with radioactive tyrosine appeared to be cis-p-coumaric acid. The product of barley TAL is exclusively the trans- isomer of p-coumaric acid (Neish, 1961). However, during the examination of the product of the Sporobolomyces enzyme, no attempt was made to avoid isomerizing conditions, and the cis- isomer could well be an artifact.

The ultraviolet spectrum of the eluted compound in 0.05N NaOH agreed closely with that of authentic p-coumaric acid, with a maximum at 333nm and a secondary peak around 304nm. The mobility of the compound distinguished it from p-hydroxyphenylpyruvic acid, which has the same ultraviolet spectrum as p-coumaric acid.

The PAL reaction with phenylalanine produced one major spot. This agreed with trans-cinnamic acid with respect to Rf in two solvent systems (Table I). A minor spot appeared to be cis-cinnamic acid and was probably a light-induced artifact.

The spectrum of the product of the PAL reaction agreed very closely with that of authentic cinnamic acid, with a maximum at 268nm.

TABLE I

COMPARISONS OF THE PRODUCTS OF TAL AND PAL REACTIONS WITH KNOWN COMPOUNDS

Compound	Rf solvent 1*	Rf solvent 2**	Colour with p-nitroaniline
trans-p-coumaric acid	.27	.41	blue
cis p-coumaric acid	.27	.71	blue
p-hydroxyphenypyruvic acid	.04	.82	pale pink
TAL reaction product (major)	.30	.43	blue
TAL reaction product (minor)	.29	.72	blue
cis-cinnamic acid	.91	.40	
trans cinnamic acid	.91	.54	
PAL reaction product (major)	.86	.45	
PAL reaction product (minor)	.86	.59	

* benzene-acetic acid-water (10:7:3), upper phase
** 2% formic acid

Partial purification of the enzymes

The percent recovery and the degree of purification of the enzymes varied from experiment to experiment. Table II summarizes the results of two typical attempts at purification. The steps referred to in the table are the purification steps described in Material and Methods.

Although recovery and specific activity of the various fractions varied, the 40-60% ammonium sulfate fraction seemed to consistently have relatively high PAL and TAL activity. The early fractions seemed to have a higher ratio of TAL to PAL activity than did the later fractions. This was noted with the TAL from wheat (Young and Neish, 1966). Ogata et al (1967b) noted that during the purification of the Rhodotorula enzyme, the ratio of

TABLE II

PARTIAL PURIFICATION OF PAL AND TAL

Step	Volume (ml)	Total protein (mg)	PAL Units*	PAL SA**	PAL Recovery	TAL Units*	TAL SA**	TAL Recovery
Experiment 1								
1. crude extract	20	284	318	1.22		111	0.309	
2. after protamine sulfate	24	151	207	1.33	66 %	129	0.612	100 %
3. ammonium sulfate								
30-40%	12	36	30	.835	9.5%	12	0.33	10.8%
40-50%	12	6.9	34.8	5.0	10.9%	15.7	2.3	14.1%
50-60%	12	3.4	32.2	9.5	10.1%	10.4	3.1	9.3%
60-70%	12	6.6	30.0	4.5	9.5%	8.5	1.3	7.7%
Experiment 2								
1. crude extract	25	212	1684	7.65		574	2.71	
2. after protamine sulfate	28.5	154	-	-	-	-	-	-
3. ammonium sulfate								
30-40%	15.4	34.7	381	11	22.6%	285	8.2	49.6%
40-50%	11.4	5.7	300	52.7	18.5%	93.5	16.4	16.3%
50-60%	15.6	2.4	51.4	21.4	31.3%	93.5	16.4	16.3%
60-70%	14.2	25.5	202	7.9	12.4%	50.4	1.6	8.8%

* one unit - the amount of enzyme which catalyzes the conversion of 0.01μmoles of phenylalanine to cinnamic acid, or of tyrosine to p-coumaric acid, in one hour at 37°.

** SA (specific activity) - units per milligram of protein

PAL to TAL did not change. In Sporobolomyces, on the other hand, there is a suggestion that the TAL protein is precipitated at lower ammonium sulfate concentrations than is PAL.

The maximum purification of PAL was 6.9 fold over activity of the crude extract; the maximum purification of TAL was 6.1 fold.

Studies on the partially purified enzyme

The 40-60% ammonium sulfate fractions were pooled and used in studies of the enzyme.

1. Determination of the Michaelis constant.

The Michaelis constant, Km, was calculated by the method of Lineweaver and Burke (1934). The Km values for PAL and TAL are 4×10^{-4}M and 2×10^{-3}M respectively (Figures 2 and 3).

These values are within the range of values of those determined for the ammonia lyases from other sources. (Table III).

The potato and corn enzymes do not follow Michaelis kinetics. At low substrate concentrations, the potato enzyme has a Km of 0.38×10^{-4} M and at high substrate concentrations, 2.6×10^{-4} M. The catalytic efficiency thus increases at higher substrate concentrations. On the basis of this Km data and of competition data, Havir and Hansen (1968b) suggest that allosteric interactions are involved. Marsh et al (1968) suggest that in corn, different isozymes with different characteristics are responsible for the varying Km.

It is quite possible that many of the enzymes do not follow Michaelis kinetics, and that more careful studies would reveal a variable Km. Neish, for example, found three values for the Km of barley TAL (1961).

Figure 2. (facing) Determination of the Michaelis constant for PAL.

Figure 3. (facing) Determination of the Michaelis constant for TAL.

FIG. 2

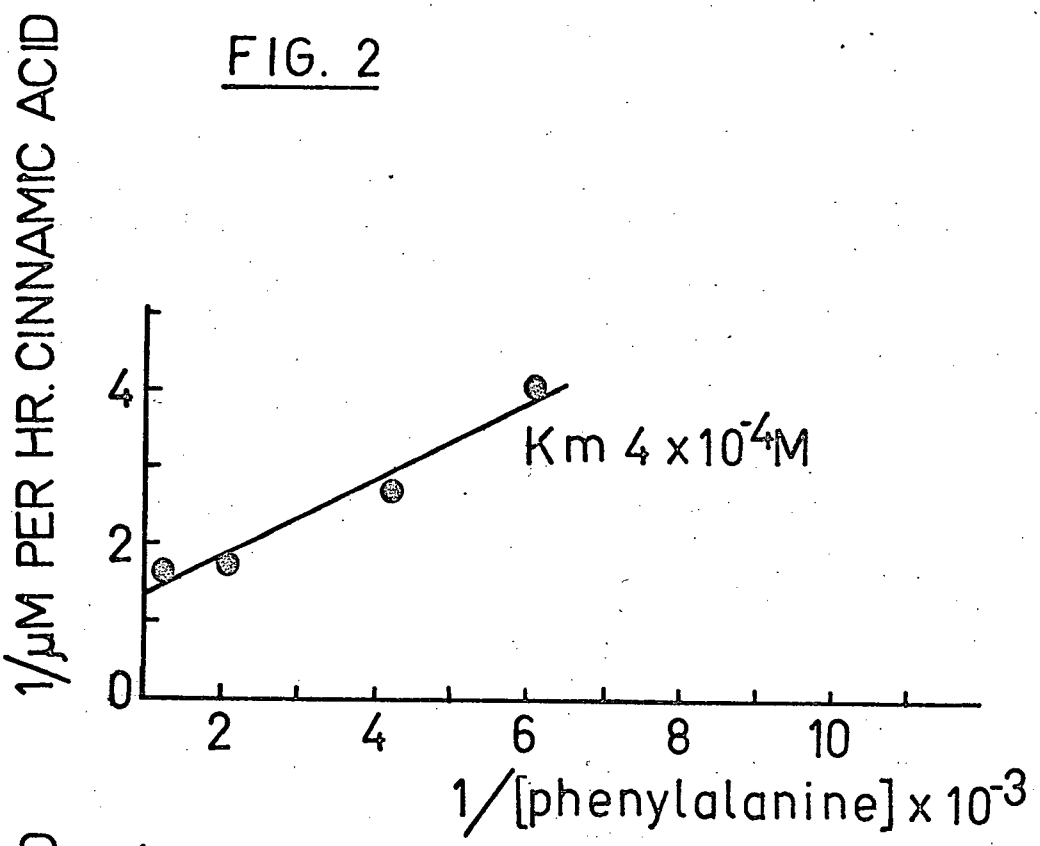

1/µM PER HR. CINNAMIC ACID
0
2
4
Km 4 x 10⁻⁴M
2
4
6
8
10
1/[phenylalanine] x 10⁻³

FIG. 3

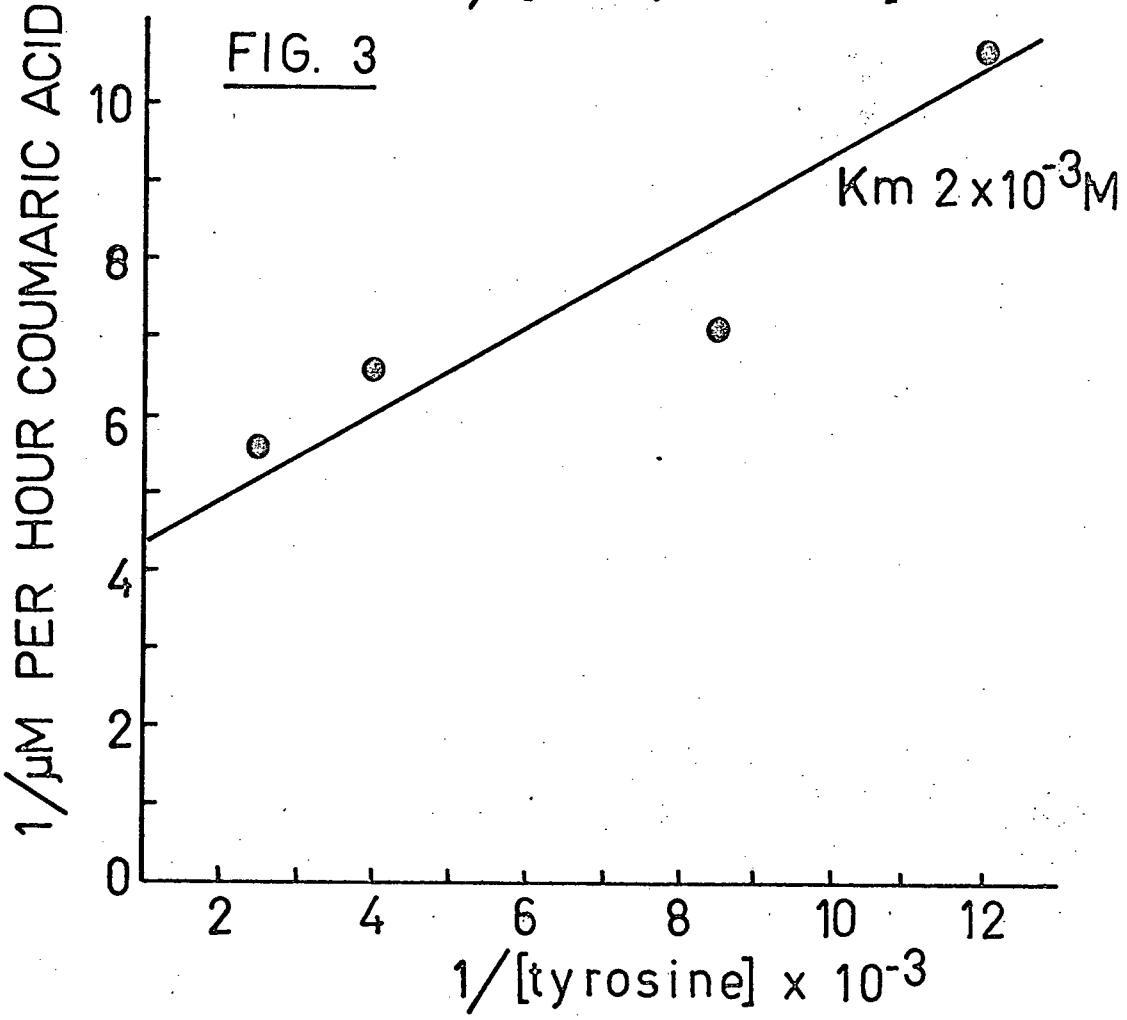

1/µM PER HOUR COUMARIC ACID
0
2
4
6
8
10
Km 2 x 10⁻³M
2
4
6
8
10
12
1/[tyrosine] x 10⁻³

TABLE III

MICHAELIS CONSTANTS FOR THE AMMONIA LYASES FROM VARIOUS SOURCES

Organism	Enzyme	Km	
barley	PAL	$1.7 \pm 0.3 \times 10^{-3}$M	Koukol and Conn (1961)
barley	TAL	0.84 0.74 } $\times 10^{-4}$M 1.0	Neish (1961)
sweet potato	PAL a	$10.1 \pm .4 \times 10^{-3}$M	Minimikawa and Uritani (1965a)
	PAL b	$9.5 \pm .9 \times 10^{-3}$M	
Rhodotorula glutinis	PAL	2.1×10^{-3}M	Ogata et al (1967b)
Rh. texensis	PAL	1.5×10^{-2}M	
Ustilago hordei	PAL	4.5×10^{-4}M	SubbaRao et al (1967)
potato	does not follow Michaelis kinetics		Havir and Hansen (1968b)
corn	does not follow Michaelis kinetics		Marsh, Havir and Hansen (1968)

2. Determination of the optimum pH.

The dialyzed 30-40% ammonium sulfate fraction was used in an investigation of the pH maxima of the deaminating enzymes. In order to test the effect of pH using only one ion, a series of borate buffers (.05M) ranging from pH 7.4 to 10.1 was used.

For the assay, about 20 units of PAL were incubated under standard conditions with 2.0 ml of .05M borate buffer at the various pH's (Figure 4). Another preparation was similarly incubated with phosphate and tris buffers at various pH's (Figure 5). About 20 units of PAL were incubated with the borate buffers only (Figure 4).

The nature of the suspending buffer seemed to exert an effect on the activity of TAL at various pH's. In borate buffer, the pH of maximum activity for PAL and TAL were 8.9 and 9.1 respectively. Activity dropped off sharply at higher pH's. In the tris buffer, on the other hand, the TAL activity did not seem so sensitive to the alkaline pH's, and the wide maximum noted by

Figure 4. (facing) Effect of pH on PAL and TAL activity (.05M borate buffers). (PAL activity -●- ; TAL activity -X-.)

Figure 5. (facing) Effect of pH on TAL activity using various buffers. (Borate buffer -▲- ; phosphate buffer -x- ; tris buffer -●-.)

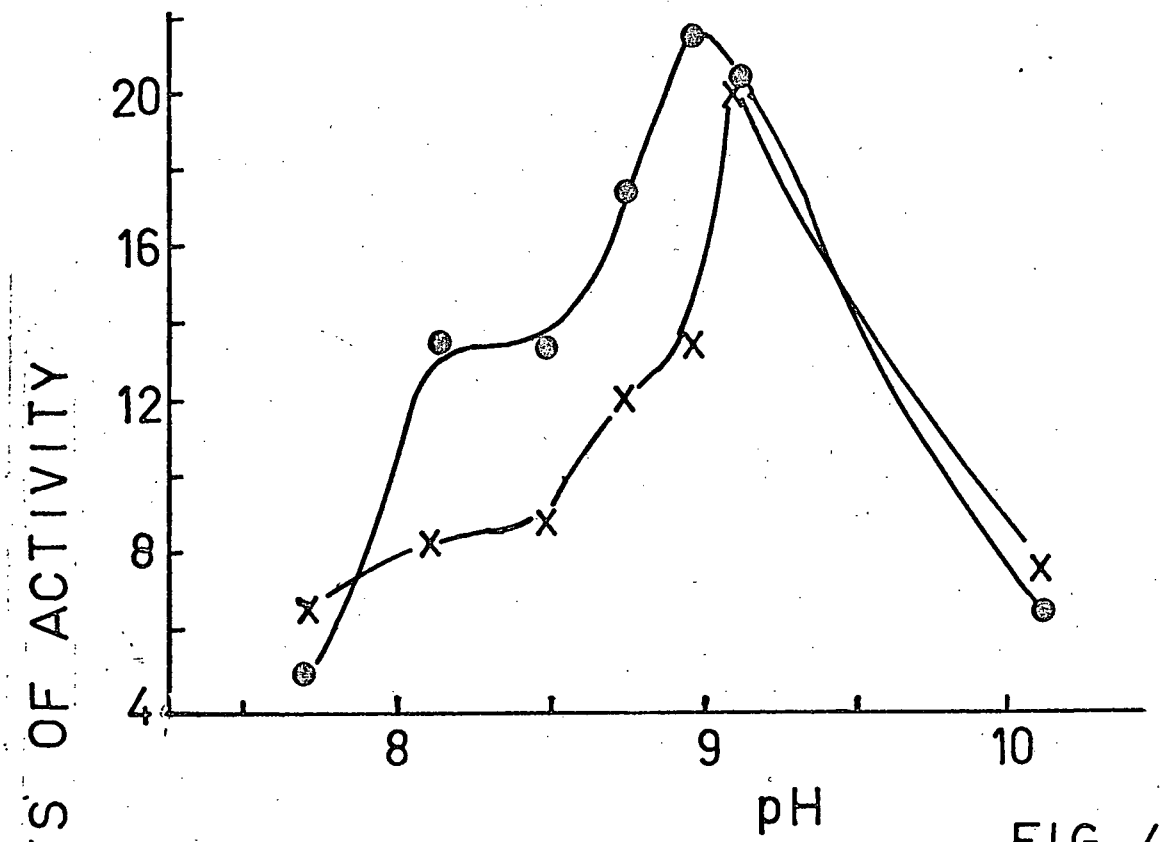

APPARENT UNITS OF ACTIVITY
20
16
12
8
4
8
9
10
pH

FIG. 4

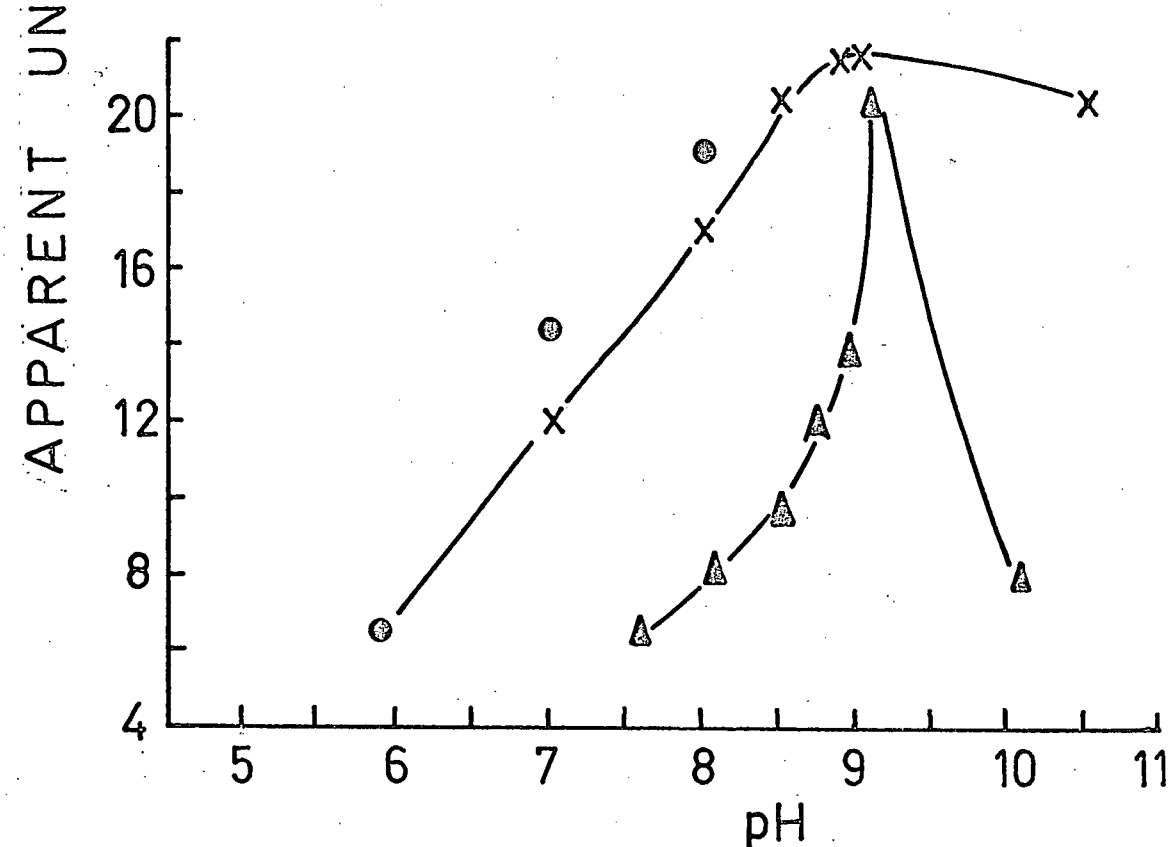

APPARENT UNITS OF ACTIVITY
20
16
12
8
4
5
6
7
8
9
10
11
pH

FIG. 5

Neish (1961) in barley PAL was evident.

The pH's of optimum activity of PAL and TAL in borate buffer were very close. Despite this, however, the shape of activity curves at various pH's were rather different for the two enzymes. Since the two enzymes behaved differently, it is probable therefore that different proteins are responsible for the deamination of phenylalanine and tyrosine.

3. Inhibition of activity

There was some evidence at this stage of the investigation that the two deaminations are catalyzed by different proteins. TAL seemed to be precipitated by lower concentrations of ammonium sulfate than PAL. Then, too, the pH curves differed in shape.

The similarity of the active sites of two enzymes can often be assessed by the degree of competitive inhibition of one reaction by the substrate of the other. If, on the other hand, one substrate does not compete with the other for the active site, the two enzyme functions are probably located on different proteins.

To assess the inhibition of the enzymes by several compounds, a modification of the usual assay was set up. To the reaction mixtures were added potential inhibitors and less than 0.2 umoles of labelled substrate. Aliquots of the ether extracts were put into vials and counted in a liquid scintillation counter.

The data shown in Table IV indicates that each enzyme is inhibited by the substrate and the product of the other enzyme. Similar situations have been discovered in some of the ammonia lyases from other sources. Koukol and Conn (1961) found that barley PAL was inhibited by trans-p-coumaric acid, L-DOPA and phenylserine, in that order. They were not able to decide whether these inhibitors acted by competition for the active sites, or by allosteric

TABLE IV

SUBSTRATE COMPETITION AND PRODUCT INHIBITION OF PAL AND TAL

Reaction mixture	cpm*	% of control
tyrosine ammonia lyase		
control (no inhibitor)	3150	
5 μmoles L-phenylalanine	845	27
4 μmoles p-coumaric acid	525	17
4 μmoles cinnamic acid	0	0
phenylalanine ammonia lyase		
control (no inhibitor)	9240	
5 μmoles L-tyrosine	4676	51
4 μmoles p-coumaric acid	1890	21
4 μmoles cinnamic acid	2900	31

*corrected for background and ether extractable material from substrate

inhibition.

Barley TAL, on the other hand, seems less sensitive to inhibition by analogues (Neish, 1961). L-Phenylalanine and meta-L-tyrosine were ineffective as inhibitors. Neish concluded that deamination of the latter two amino acids in barley must be carried out by other active sites than that which deaminates L-tyrosine.

In the case of wheat PAL, L-, DL-, and D-tyrosine are strong inhibitors (Young and Neish, 1966). On the other hand, the TAL in wheat is not very sensitive to L-phenylalanine. It would appear that the PAL and TAL functions do not share the same active site, since they are not mutually competitive. Young and Neish postulate two ammonia-lyases, one of which is quite specific for tyrosine, and the other which will deaminate phenylalanine and a large

variety of compounds. It is the latter enzyme which is subject to competition by other substrates.

PAL from sweet potato could be separated into two active fractions (Minimikawa and Uritani, 1965b). One of the proteins was found to be inhibited competitively by trans-cinnamic acid, while the other protein was subject to competitive inhibition by cinnamic acid, p-coumaric acid, and caffeic acid. p-Fluorophenylalanine and L-tyrosine were also competitive inhibitors.

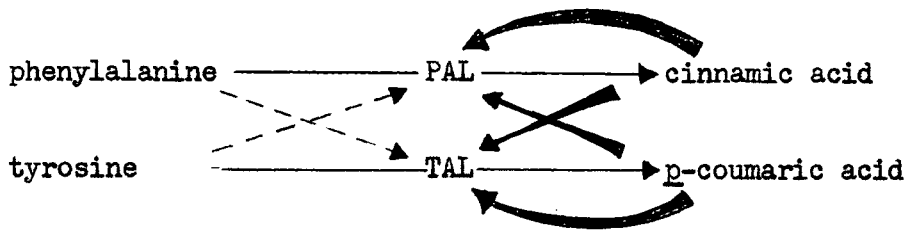
In the case of the Ustilago PAL, strong end-product inhibition was noted (SubbaRao et al, 1967). Cinnamic acid was a strong inhibitor, but p-coumaric acid is not. This was in contrast to the situation observed in the Sporobolomyces enzyme, and in barley PAL, which was more inhibited by p-coumaric acid than by cinnamic acid (Koukol and Conn, 1961). The Ustilago enzyme was affected by a wide variety of compounds, but L-tyrosine, which was not deaminated by this enzyme, did not inhibit the activity at all.

PAL from potato was subject to competitive inhibition by D-phenylalanine (Havir and Hansen, 1968b). The enzyme was also inhibited by cinnamic acid. Interestingly enough, maximum inhibition by the end-product did not occur at the pH of maximum enzyme activity.

The enzymes from Sporobolomyces roseus appeared to be very closely related. Not only did each substrate interfere with the other enzyme, but also the product of each reaction inhibits both that enzyme and the other enzyme (Figure 6).

The two enzymes seemed to be subject to end-product inhibition. But the reaction inhibited by an end-product is generally the first one leading solely to that end-product. In this case, the PAL reaction, which did not produce p-coumaric acid, was inhibited by it. Similarly, TAL is inhibited by cinnamic acid.

Several explanations can be offered. Perhaps the two enzymes were one protein with wide specificity. In that case, the active site would not have recognized the difference between phenylalanine and tyrosine, or between cinnamic acid and p-coumaric acid.



Heavy lines indicate inhibition

Dashed lines indicate substrate competition

Figure 6. Substrate competition and product inhibition of PAL and TAL

On the other hand, this phenomenon might not have been end-product inhibition, strictly speaking. Cinnamic acid inhibits several plant enzymes such as phenolase and p-coumaric acid hydroxylase (Zucker et al, 1967). Cinnamic acid may have exerted a general inhibitory effect on many enzymes not necessarily on the same biosynthetic pathway, rather than specifically inhibiting PAL and TAL.

There is still another possibility. The reaction inhibited by an end-product is generally the first one leading solely to that end-product. This mechanism permits the regulation of production of one end-product without interfering with the synthesis of other products of the metabolic pathways. If cinnamic acid and p-coumaric acid were needed at the same rate, then this mutual inhibition would be a very efficient mechanism to prevent excess of

one or the other. On the other hand, if the p-coumaric acid and cinnamic acid were not needed at the same rate, but were interconvertible, then the reactions inhibited by the end-products are effectively equivalent to reactions leading directly to that end-product. While there is no evidence to suggest that p-coumaric acid and cinnamic acid are interconvertible in Sporobolomyces, the hydroxylation of benzoic acid in this organism is well established (Moore et al, 1967). It would be interesting to know if benzoic acid and hydroxybenzoic acid inhibit PAL and TAL in Sporobolomyces roseus, as they do in Ustilago (SubbaRao et al, 1967).

Growth of Sporobolomyces roseus

Sporobolomyces roseus is relatively undiscriminating with respect to carbon and energy source. Cells were found to grow in media containing only salts and a phenolic compound as sole carbon source (Table V).

Although cells would grow on a wide variety of carbon sources, as shown in Table V, no growth could be demonstrated of cells inoculated into medium containing cinnamic acid. It appeared possible that the enzymes responsible for the degradation of cinnamic acid were inducible by a compound other than cinnamic acid. Degradation of phenylalanine occurs via cinnamic acid; presumably cells grown on phenylalanine as a sole carbon source would possess all the enzymes required to degrade cinnamic acid. Yet even cells that had been growing rapidly in phenylalanine stopped growth when placed in the cinnamic acid medium. Although the cells were able to use phenylalanine as a carbon source, they could not utilize cinnamic acid. This could be because they lacked a transport system for the compound, or because the high cinnamic acid concentration inhibited the activity of some enzyme.

TABLE V

UTILIZATION OF VARIOUS COMPOUNDS AS CARBON SOURCE BY SPOROBOLOMYCES ROSEUS

Compound	Concentration	Growth (optical density at 600nm after 48 hours)
p-coumaric acid	.05%	1.85
p-hydroxyphenylpyruvic acid	.05%	ca. 1.0
phenylpyruvic acid (sodium salt)	.05%	0.82
p-hydroxybenzoic acid	.05%	1.10
ferulic acid	.05%	ca. 1.0
vanillic acid	.05%	"
caffeic acid	.05%	"
glucose	1.0 %	3.5
L-tyrosine	.05%	1.1
L-phenylalanine (sole carbon and nitrogen source)	.5 %	1.2
cinnamic acid*	.05%	)
	.01%	) no growth
(sodium salt)	.05%	)

*see text for discussion

On the other hand, when a 48 hour culture that had been grown on 1% glucose was resuspended in 0.05% cinnamic acid, the optical density of the culture increased by nearly 40% in 24 hours. In some way, the larger inoculum of cells was able to grow in medium in which a small inoculum could not grow.

Production of enzymes during growth of a batch culture

Growth, as followed by changes in optical density, generally followed the sigmoid curve typical of batch cultures. If, however, the inoculum was of

cells in the logarithmic phase of growth, the lag phase was abbreviated or absent.

Cell number and optical density increased logarithmically for about 24 hours; then the rate of division slowed (Figure 6). The optical density reached a maximum after about 48 hours, after which it remained the same for several days, or slowly decreased. This period was marked by the formation of oil droplets in the cells, and by rapid budding in chains or the formation of pseudomycelia.

The enzyme activity of the cells (expressed as activity per milligram of protein in buffer extracts) did not remain constant during the growth of the culture. The activity reached a peak just after the culture had entered the stationary phase of growth (Figure 6). During the logarithmic phase of growth, the specific activity was low but rising; during stationary phase, high but falling. This pattern was consistent from one experiment to another although the actual values of enzyme activity varied slightly, as shown in Figures 6 and 7. A considerable degree of uniformity existed between cultures inoculated with identical cells and grown at the same time. Such cultures are very similar both with respect to cell number and enzyme content. A typical example is shown in Figure 8. In this experiment flasks were given identical inocula and were allowed to grow for 48 hours. At the end of this time the cells from each flask were resuspended in a different replacement medium. The optical density data plotted on a logarithmic scale indicated that the cells did not begin to grow immediately in the new medium. During the lag, the enzyme content was that characteristic of the previous medium. Thus, even up to 53 hours after the original inoculation, the cells were behaving in a similar fashion. After this time, the enzyme production reached levels characteristic of the replacement medium.

Figure 6. (facing) Enzyme production and increase in cell number in Sporobolomyces roseus grown on minimal medium (1% glucose); Expt. 1. (PAL activity—●— ; TAL activity—■—; optical density--×-)

Figure 7. (facing) Enzyme production and increase in cell number in Sporobolomyces roseus grown on minimal medium (1% glucose); Expt. 2. (PAL activity—●—; TAL activity —■—; optical density --×-)

FIG. 6

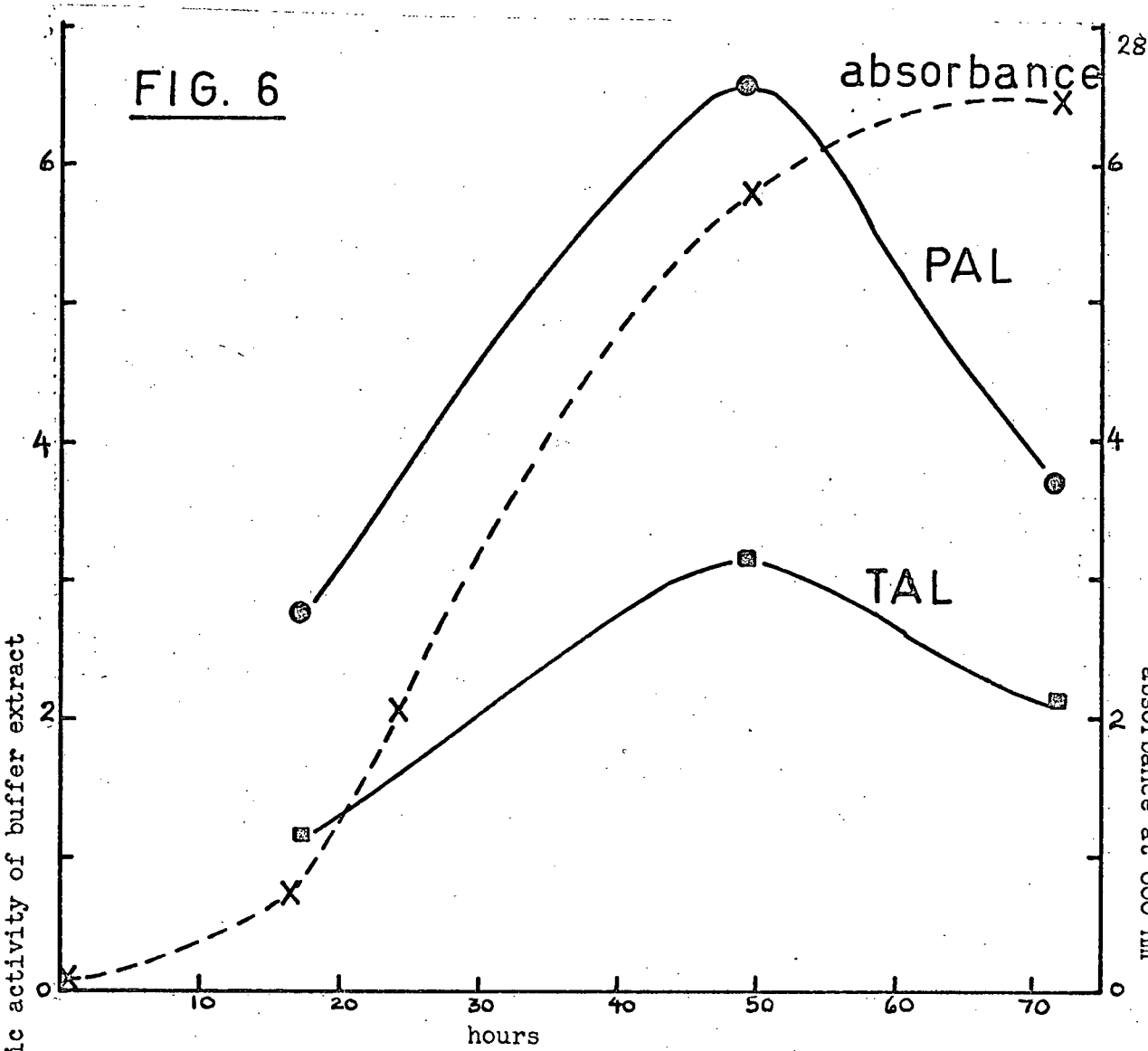
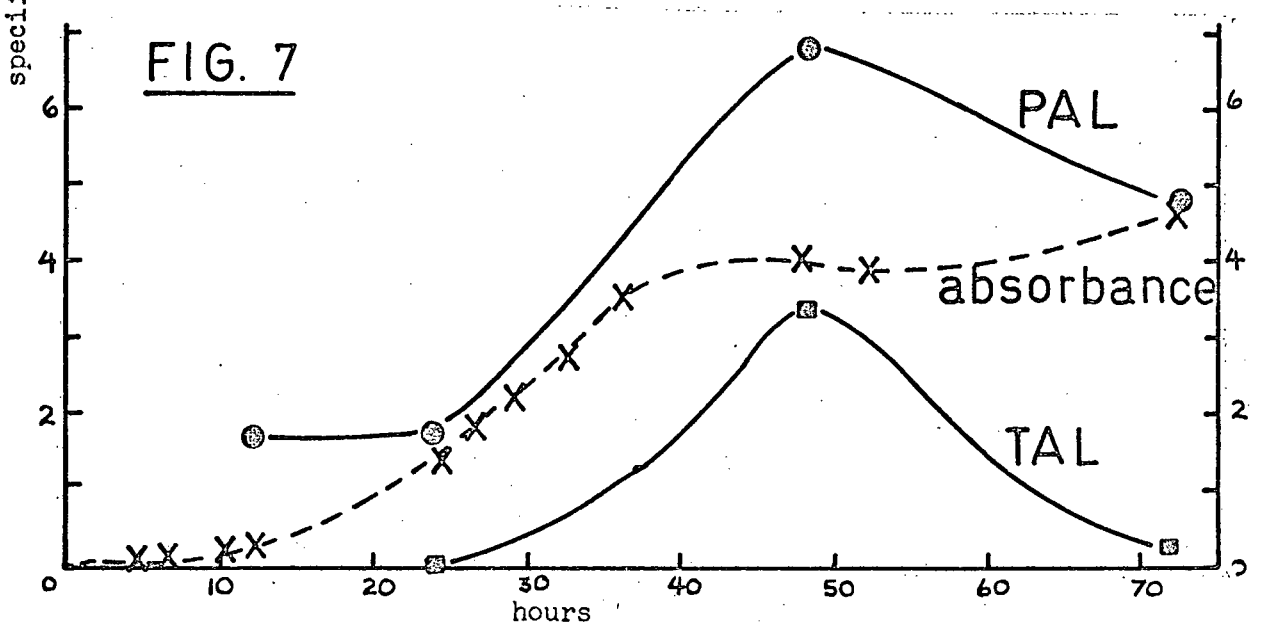

absorbance
PAL
TAL
specific activity of buffer extract
absorbance at 600 nm
hours
0
2
4
6
10
20
30
40
50
60
70

FIG. 7


PAL
absorbance
TAL
hours
0
2
4
6
10
20
30
40
50
60
70

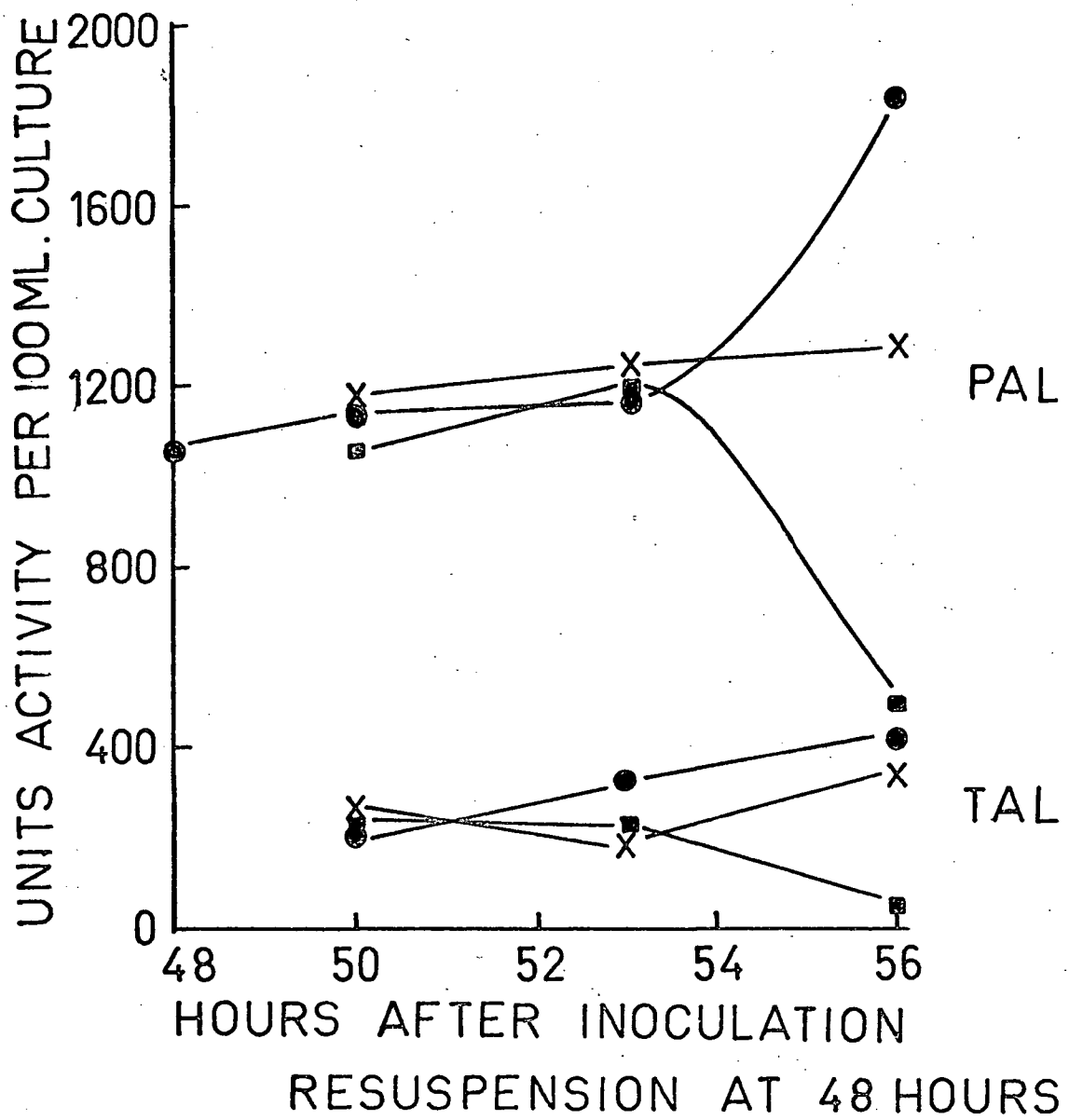


FIG. 8

Enzyme content of cultures inoculated at the same time and resuspended in different media after 48 hours growth. (glucose replacement medium —●—; p-coumaric acid replacement medium —X—; cinnamic acid replacement medium —▣— .)

In a batch culture of a microorganism, the shift of growth from the logarithmic phase of growth to the stationary phase is often correlated with the exhaustion of some nutrient (Lamanna and Mallette, 1965). There are, however, cases in which this shift of growth cannot be directly ascribed to exhaustion of any of the nutrients in the medium, nor to a buildup of toxic products. This situation has been described in Penicillium urticae and a correlation between growth phase and metabolic activity noted (Bu'Lock, 1965). In the related P. islandicum, the switch in metabolic activity is correlated with exhaustion of ammonia in the medium (Gatenback and Sjoland, 1965).

If the same situation occurred in Sporobolomyces roseus as in P. islandicum, then the ammonia concentration of the medium should reach a minimum early in stationary phase. If ammonia, one of the products of the ammonia lyase, were acting as a feedback repressor of the synthesis of the enzymes, then enzyme synthesis should be expected to begin when ammonium concentration is low. Feedback repression of enzyme synthesis by an ion has been noted before, as for example, in E. coli, in which phosphate ion inhibits the production of alkaline phosphatase (Torriani, 1960).

The role of carbon and nitrogen levels in the production of PAL and TAL

To determine what role is played by the ammonia nitrogen levels in the medium, cells were grown in media that varied with respect to relative ammonium concentration. This was accomplished by varying solutions of the same mineral salts concentration, but of different glucose concentrations. A medium that is only 0.1% with to glucose is relatively rich in ammonia. Similarly, a medium that is 2% in glucose is ammonia-poor.

The growth of the cells and the utilization of glucose and ammonia

nitrogen is shown in Figures 9, 10 and 11. The cells grown in 1% and in 2% glucose multiplied at the same rate. Cell multiplication and utilization of nitrogen slowed after 35 hours of growth. At this time, both ammonia nitrogen and glucose were still present in the medium.

In the case of the cells grown on 0.1% glucose, increase in cell number was very slow. The level of glucose in the medium fell below the range of the test, but the ammonia levels remained at two thirds of the original concentration.

The results indicate that ammonia nitrogen levels were fairly high in all three cases up to 72 hours after inoculation. Since after 72 hours' growth, ammonia nitrogen was still present at one-quarter to two-thirds the original concentration, and since both the 1% and the 2% glucose cultures entered the stationary phase of growth at the same time, the limiting nutrient appeared to be something other than ammonia.

In the low (0.1%) glucose culture, the rate of growth was slower, but the switch into stationary phase occurred at approximately the same time as in the cultures grown in higher concentrations of glucose.

The specific activities of the ammonia lyases in the cells varied among the three cultures (Figures 7, 12 and 13). Cells grown on 2% glucose yielded enzyme preparations with very low specific activities, while the cells grown on 0.1% glucose gave preparations with high specific activities.

It can be concluded from this experiment, that if a limiting concentration of some nutrient causes growth to enter the stationary phase, it is not carbon nor ammonia, nor do lowered levels of ammonia appear to de-repress the formation of the ammonia lyases. The triggering factor is one of the other mineral nutrients, or an internal, accumulated product.

The above experiment shows that the cells grown on high (2%) glucose

Figure 9. (facing) Glucose and ammonia utilization by Sporobolomyces roseus cells grown on minimal medium with 2% glucose. (optical density--x-; glucose content—o—; ammonia content···▲··)

Figure 10. (facing) Glucose and ammonia utilization by Sporobolomyces roseus cells grown on minimal medium with 1% glucose. (optical density--x-; glucose content—o—; ammonia content···▲··)

Figure 11. (facing) Glucose and ammonia utilization by Sporobolomyces roseus cells grown on minimal medium with 0.1% glucose. (optical density-x- ; glucose content—o—; ammonia content···▲··)

FIG. 9

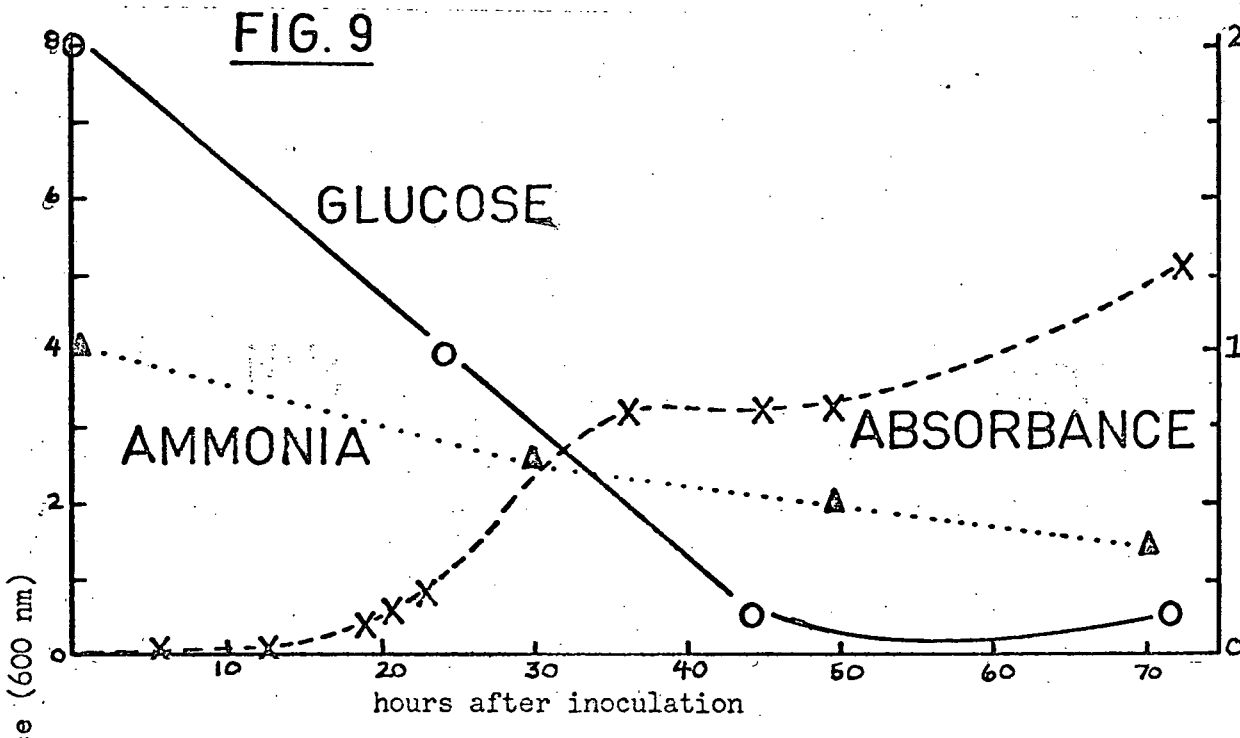


FIG. 10

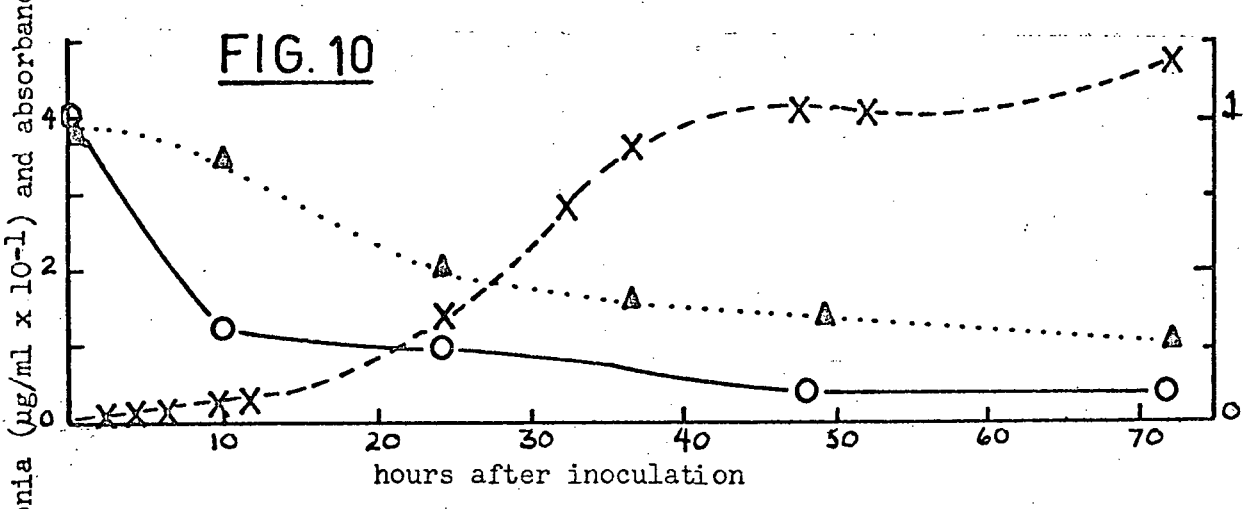


FIG. 11

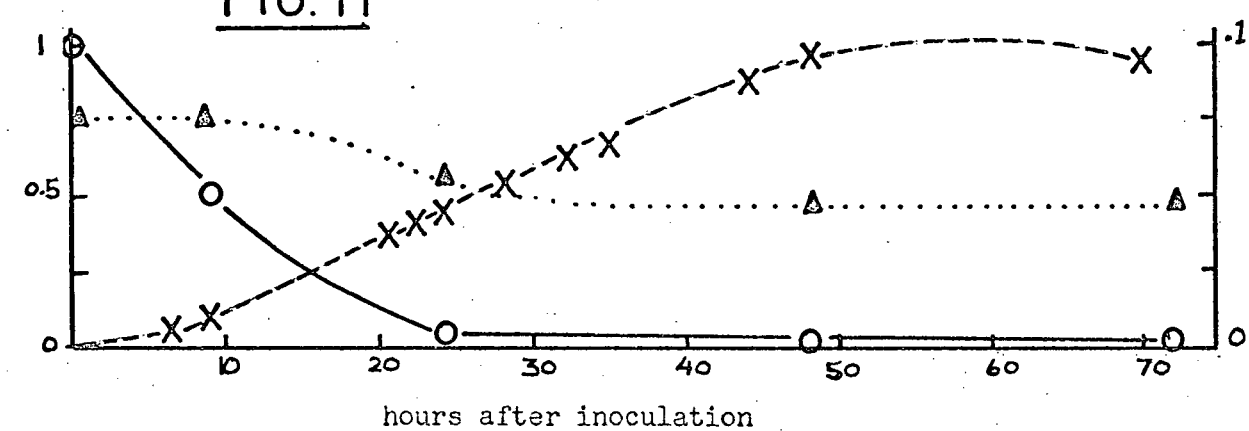


Figure 12. (facing) Growth and enzyme production of Sporobolomyces roseus cells growing on salts and 2% glucose. (optical density --x- ; PAL activity —●— ; TAL activity -■-)

Figure 13. (facing) Growth and enzyme production of Sporobolomyces roseus cells growing on salts and 0.1% glucose. (Note that the optical density scale is different than that in Figures 7 and 12). (optical density--x-; PAL activity —●— ; TAL activity -■-)

FIG. 12

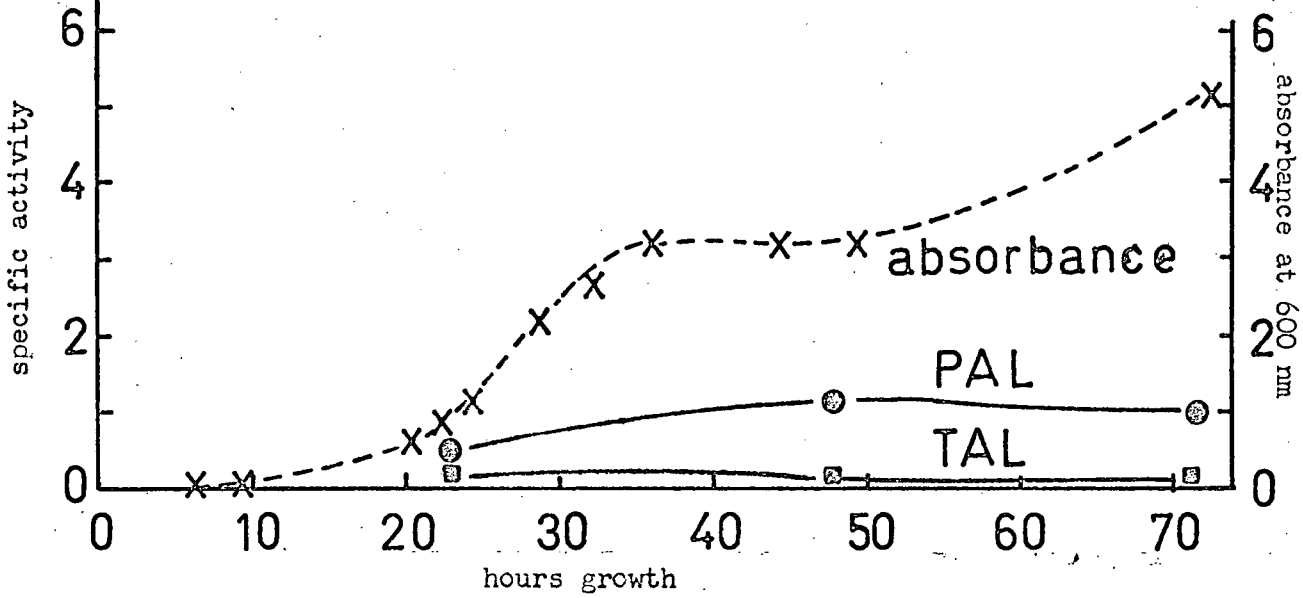
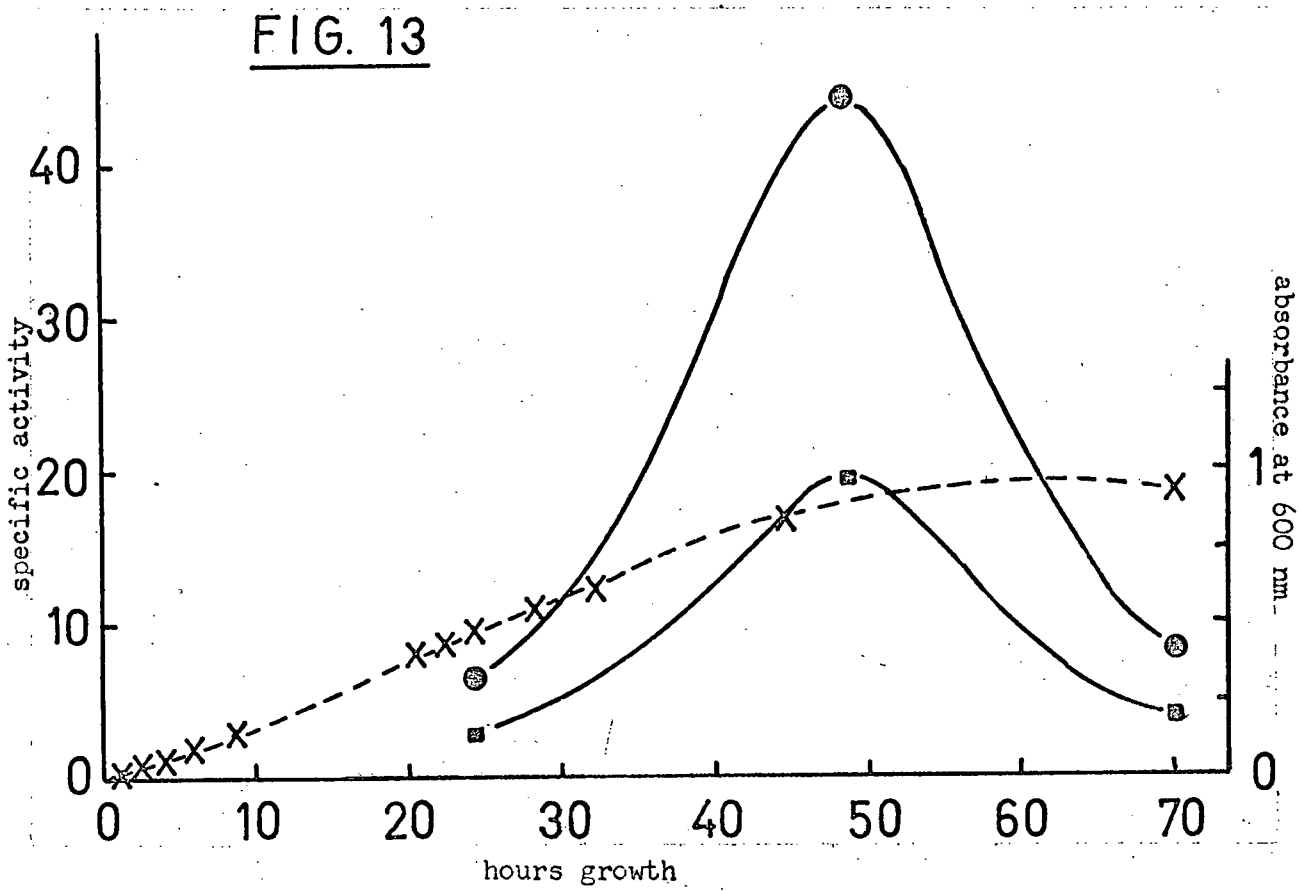

specific activity
absorbance at 600 nm
absorbance
PAL
TAL
hours growth
0
2
4
6
0
10
20
30
40
50
60
70

FIG. 13


specific activity
absorbance at 600 nm
hours growth
0
10
20
30
40
0
1
0
10
20
30
40
50
60
70

had almost no ammonia lyases, while those grown under conditions of low (0.1%) glucose contained very high levels of the enzyme. A similar repression of catabolic enzymes by high levels of carbon and energy sources in the medium has often been noted in microorganisms (McFall and Mandelstam, 1963; Loomis and Magasanik, 1964). Such repression has been most commonly associated with induced enzymes.

The repression of the enzyme is felt to be specifically produced by an end-product of the enzyme action, or some closely related compound. Glucose acts as a repressor only when it can give rise to sufficient amounts of the specific regulator (McFall and Mandelstam, 1963). Although several theories have been put forward (Loomis and Magasanik, 1964; Adhya and Echols, 1966; Stokes and Powers, 1967), the precise nature of the cause of the glucose effect is unknown. Most reports, however, discuss the phenomenon as a repression of inducible enzymes. Since PAL and TAL appear in cells that have been grown on glucose and salts alone, they are certainly not inducible enzymes, although they may be subject to catabolite repression.

Production of enzyme by cells growing in the presence of substrate

While inducible enzymes are produced only in the presence of the inducer, (usually the substrate), even the production of constitutive enzymes may be modified by environmental influences. For example, bacterial cells grown on pyruvate as a carbon source ferment glucose at a slower rate than if grown with glucose in the medium (Breever, Mickleson and Werkman, 1943).

To examine the possible stimulation of TAL and PAL by substrate, Sporobolomyces roseus cells were grown under the usual conditions in minimal medium that contained additional tyrosine at levels up to 0.1%.

In Sporobolomyces roseus, tyrosine added to the glucose-salts medium increased the production of both TAL and PAL (figure 14). Figure 15 shows that at high levels of tyrosine, the stimulation of the production of the enzymes is proportionally lower than at the lower levels. There is a possibility that at these levels, the tyrosine is acting as an additional carbon source, since after the initial deamination, the whole of the carbon skeleton is available for use by the organism. On the other hand, the production of the enzymes by the cell may be approaching the highest level of which the cell is capable.

Figure 15 demonstrates that the presence of the TAL substrate stimulates the production of both enzymes. However, the presence of high levels of tyrosine causes an increase in the relative amount of TAL formed, suggesting that two separate proteins are involved, but that the production of the two proteins is controlled by the same system.

Replacement media studies

Sporobolomyces roseus cells were grown in minimal medium, then harvested aseptically at 48 hours and resuspended in salts plus .05% p-coumaric acid, .05% cinnamic acid, or .05% glucose as control. The low concentration of carbon sources were used in order to avoid any possible confounding effects by high levels of energy source.

All cultures experienced a two-hour lag period, then began to grow again. The cells resuspended in the p-coumaric acid medium grew as well as those in the .05% glucose. Although small inocula of cells introduced into medium containing cinnamic acid did not grow, the cells resuspended in .05% cinnamic acid grew as well as those in glucose or in p-coumaric acid. Whether

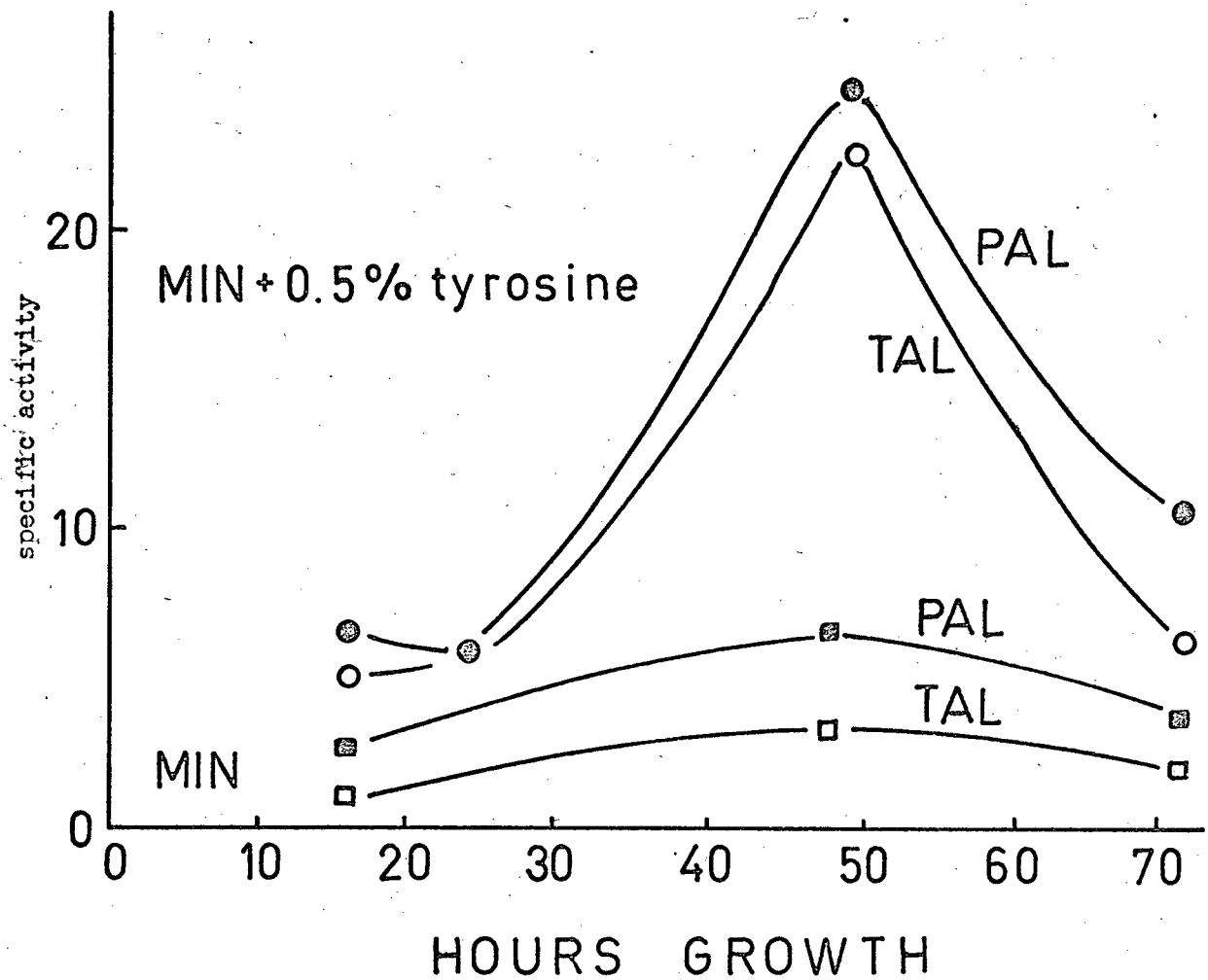


FIGURE 14

Growth and enzyme production of cells grown on minimal medium plus .05% tyrosine.

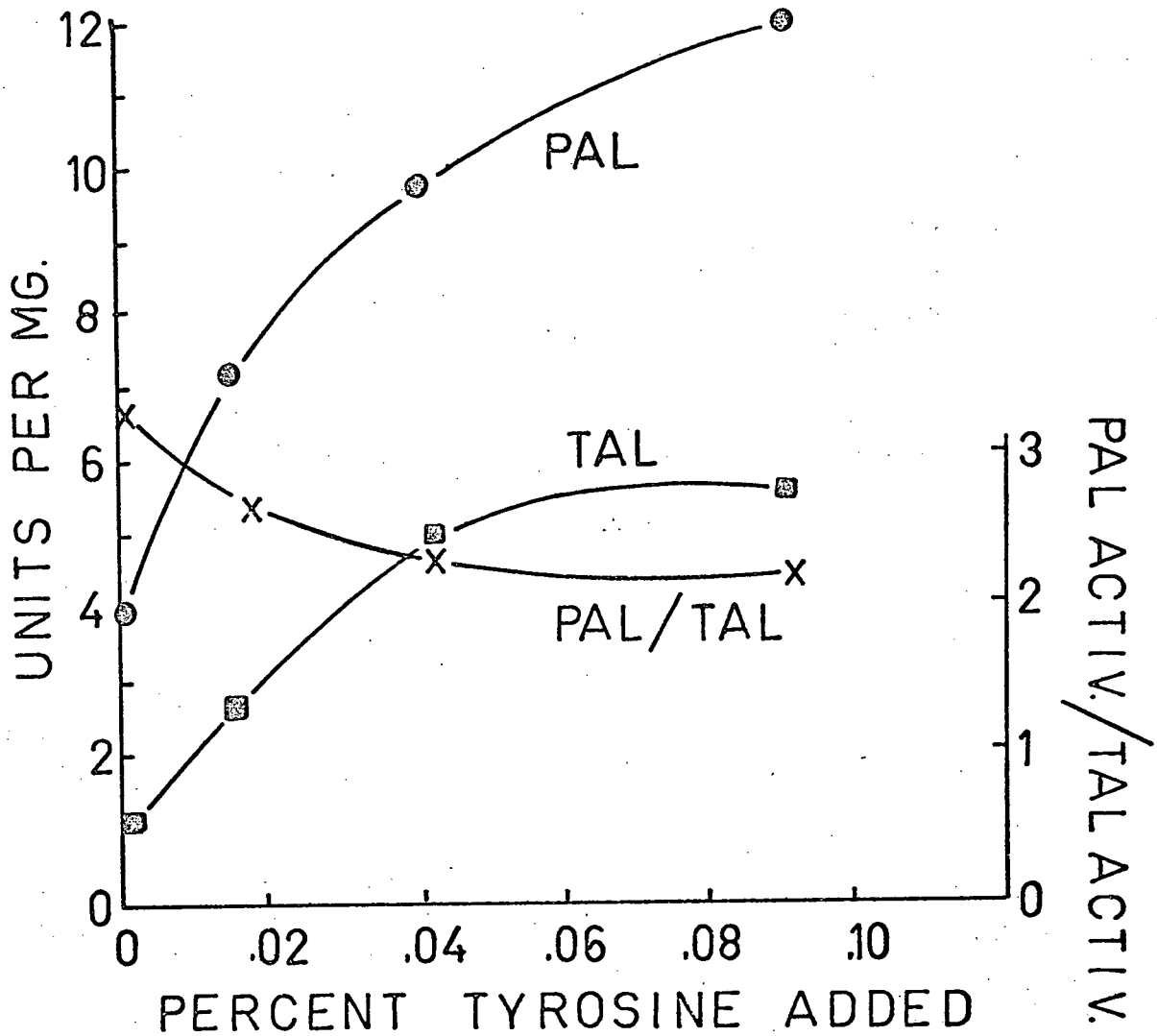


FIGURE 15

Enzyme production and ratios of specific activities in 48 hour cells grown on minimal medium with various levels of tyrosine.

this increase in cell number represents assimilation of the cinnamic acid supplied, or utilization of endogenous reserves was not determined. In either event, cinnamic acid entered the cells, as ether extracts of the washed cells showed. This high endogenous cinnamic acid content was taken into account in the calculation of the PAL content of the cells.

The enzyme activity of the cells resuspended in .05% glucose remained constant for five hours, then reached a maximum level as the cultures entered early stationary phase (Figure 16a). The enzyme activity of the cells resuspended in p-coumaric acid rose to a level which remained constant (Figure 16b). It is difficult to correlate the behaviour of the enzyme levels with the growth of the culture.

For the first five hours after resuspension, PAL and TAL levels in the cells supplied with cinnamic acid were the same as the levels in the glucose-supplied cells (Figure 8 and Figure 16c). After this time, however, the enzyme content of the former cells dropped sharply; TAL activity completely disappeared. By 24 hours the levels had risen to the levels of the glucose-supplied controls.

A similar experiment was set up to study the time course of enzyme development by cells in the presence of phenylalanine and tyrosine. Sporobolomyces roseus cells were grown for 48 hours in minimal medium containing 1% glucose. The cells were harvested aseptically and resuspended in salt with the following carbon sources: 1% glucose, 1% glucose plus .05% tyrosine, and 1% glucose plus .05% phenylalanine. Absorbance data indicate a two- to four-hour period of very slow division (Figure 17). Eventually, all three cultures resumed growth.

It has been previously shown that the amount of enzyme per milligram of protein varied during the growth of the culture. In the present experiment,

Figure 16. (facing) Growth and enzyme content of Sporobolomyces roseus cells in various replacement media. A. .05% glucose plus salts; B. .05% p-coumaric acid plus salts; C. .05% cinnamic acid plus salts.
(PAL activity —●— ; TAL activity —■— ; absorbance --x-)

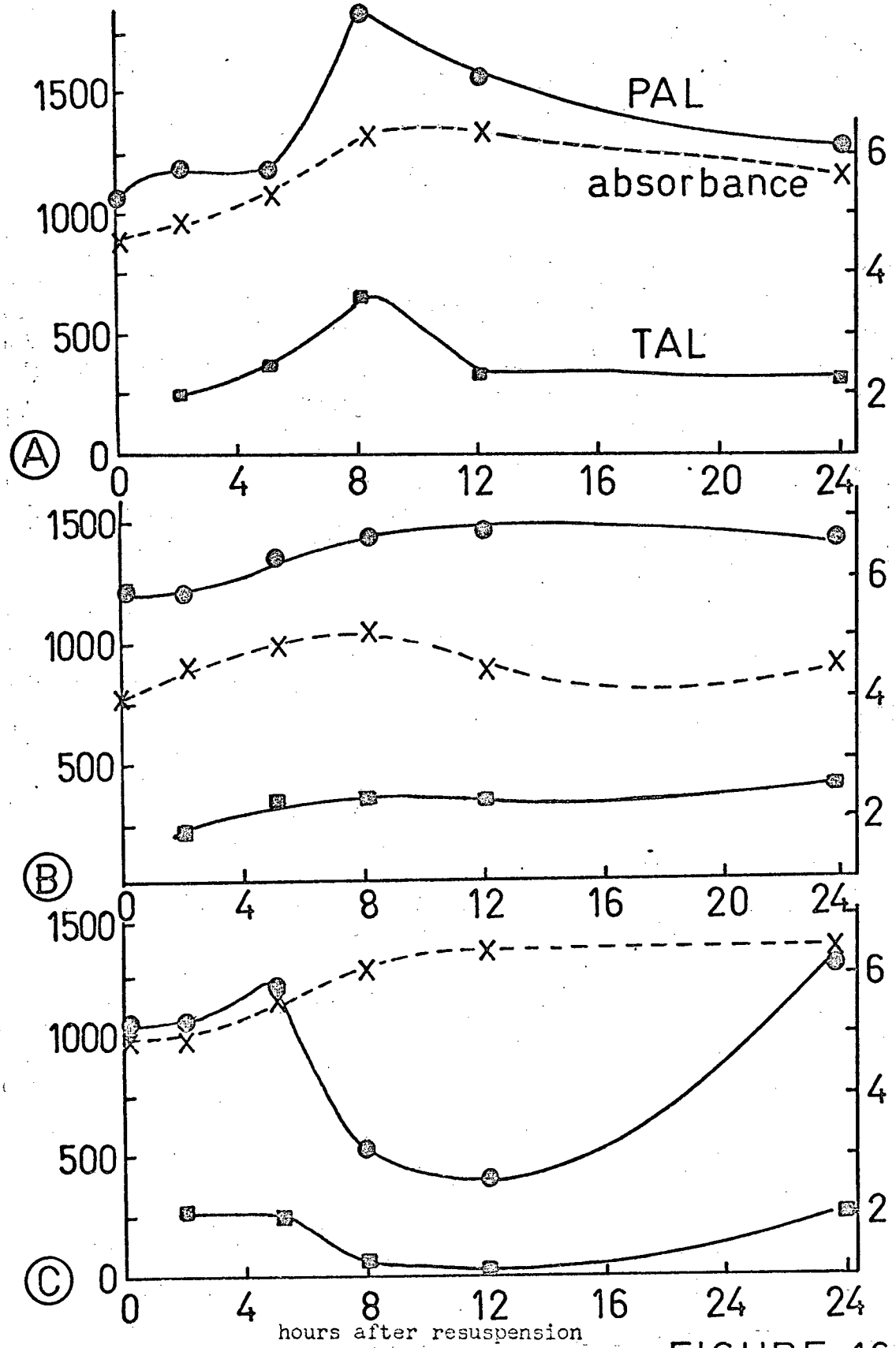

PAL
absorbance
TAL
A
B
C
1500
1000
500
0
6
4
2
0
4
8
12
16
20
24
units of enzyme in 100 ml culture
absorbance at 600 nm
hours after resuspension

FIGURE 16

Figure 17. (facing) Growth and enzyme activity of Sporobolomyces roseus cells in replacement media plus glucose. A. minimal medium; B. minimal medium plus .05% tyrosine; C. minimal medium plus .05% phenylalanine.
(PAL activity —●— ; TAL activity —■— ; absorbance—×—)

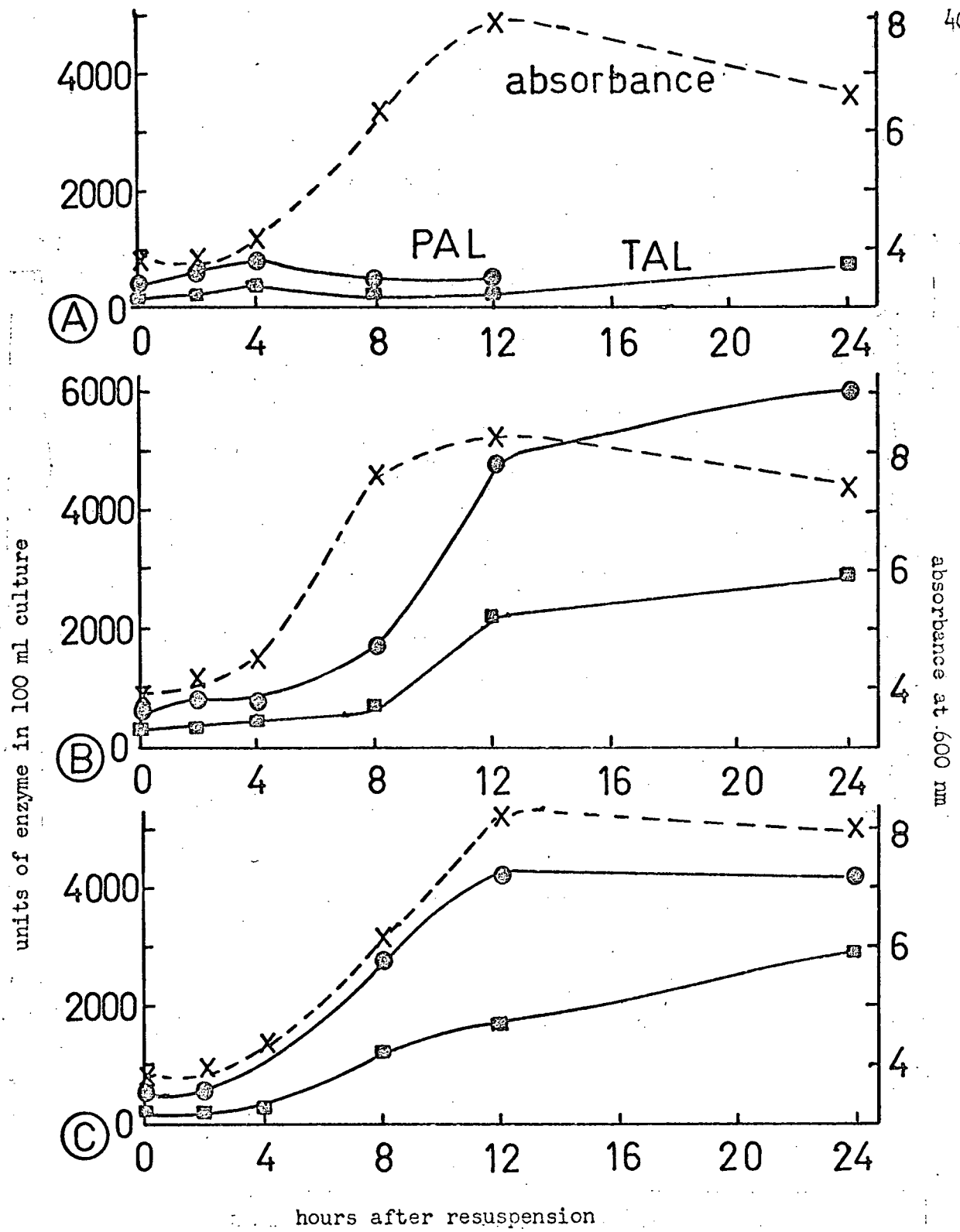

absorbance
PAL
TAL
A
B
C
4000
2000
6000
0
4
8
12
16
20
24
6
units of enzyme in 100 ml culture
absorbance at 600 nm
hours after resuspension

FIGURE 17

the enzyme content was measured of acetone powders prepared from the cells in measured volumes of the culture. Thus, activity was not expressed per milligram of protein in a buffer extract, but instead as the activity in a sample of the growing culture.

As a control, cells were resuspended in 1% glucose, the same medium as that in which they were grown (Figure 17a). The enzyme levels did not change significantly during the 24 hours of the experiment.

The levels of PAL and TAL rose in cells resuspended in glucose plus tyrosine (Figure 17b). The TAL level increased to ten times that of the glucose control, and the PAL level to 15 times that of the control. The maximum production of the enzymes occurred as the cultures entered the stationary phase of growth.

Very high levels of both enzymes were also observed in the culture with the added phenylalanine (Figure 17c). The TAL level was seven times that of the control, and the PAL level, 11 times. In this culture, the increase in enzyme content paralleled very closely the increase in cell number.

Since optical density is a linear function of cell number, the ratio of enzyme units in a volume of medium divided by the optical density of the culture provides a measure of the amount of enzyme per cell. It can be seen in Figure 18 that the rise in total enzyme content of the culture is not merely due to an increase in the total number of cells; the amount of enzyme per cell increases also.

In short, either aromatic amino acid stimulated the production of both ammonia lyases in these cells. During the growth of the culture, the enzyme content increased in the whole culture, in each cell, and as a fraction of the total cell protein.

The ratio of the two enzymes is not constant over the 24 hour period

Figure 18. (facing) Enzyme production per cell in resuspension media. A. minimal medium; B. minimal medium plus .05% tyrosine; C. minimal medium plus .05% phenylalanine. (absorbance --×- ; PAL activity per cell —●— ; TAL activity per cell —■—)

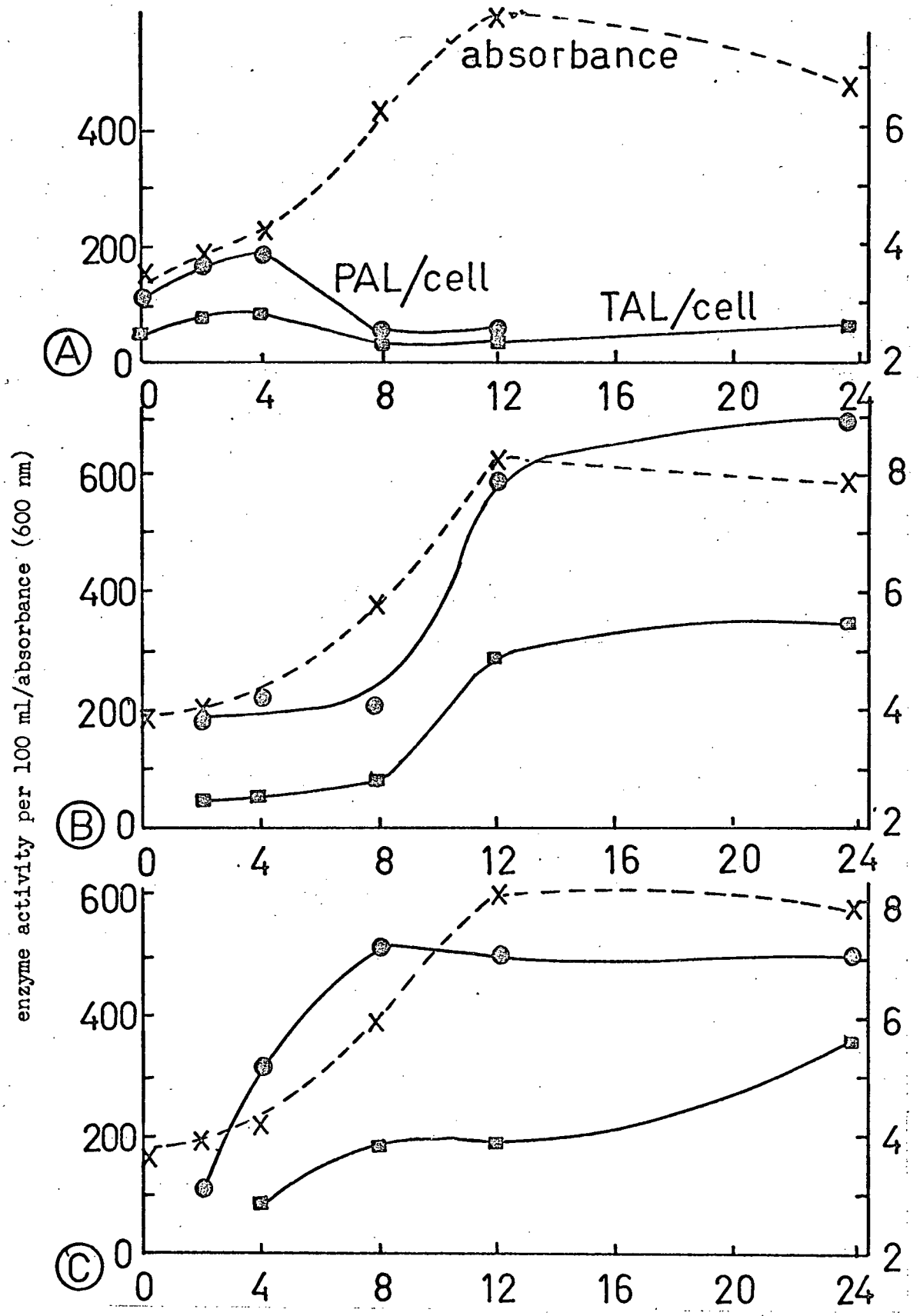

absorbance
PAL/cell
TAL/cell
A
B
C
400
200
0
600
6
4
2
8
0
4
8
12
16
20
24
enzyme activity per 100 mL/absorbance (600 nm)
absorbance at 600 nm
hours after resuspension

FIGURE 18

(Figure 19). Cells suspended in minimal medium multiply rapidly in the first 12 hours after resuspension, and during this period, the ratio of PAL to TAL is relatively high. As rapid cell multiplication continues, however, the ratio becomes smaller, i.e., there is an increase in the proportion of TAL formed.

The same general pattern is observed in cells fed glucose plus phenylalanine. In stationary phase, the PAL/TAL ratio approaches the low value characteristic of cells grown on glucose alone.

The behavior of the tyrosine-fed cells again follows the same general pattern. However, in these cells the relative amount of TAL is lower than in cells grown on glucose alone. Data from a previously described experiment show that after 48 hours growth (to early stationary phase) on 1% glucose plus .05% tyrosine, the PAL/TAL ratio is 2:1 (Figure 15). This agrees with the present value of 2:1 in cells in stationary phase.

In all three media the PAL/TAL ratio is highest during the first four hours after resuspension. This period is one of non-logarithmic cell multiplication. In all three media, it is during logarithmic growth of the culture that the PAL/TAL ratio becomes lower. This suggests that while the total enzyme protein, and the relative amounts of each enzyme produced, can be varied by external conditions, there are certain endogenous controls over the rate of production of the two proteins. During logarithmic growth of the culture, while the rate of synthesis of both proteins increases, the acceleration of TAL synthesis is greater than that of PAL. During stationary phase, the production of both proteins stops.

Experiments similar to these were carried out by Ogata *et al*, using the yeast *Rhodotorula* (1967a). *R. texensis* produces PAL even when grown on glucose alone; *R. glutinis* produces PAL only when grown on malt medium or in

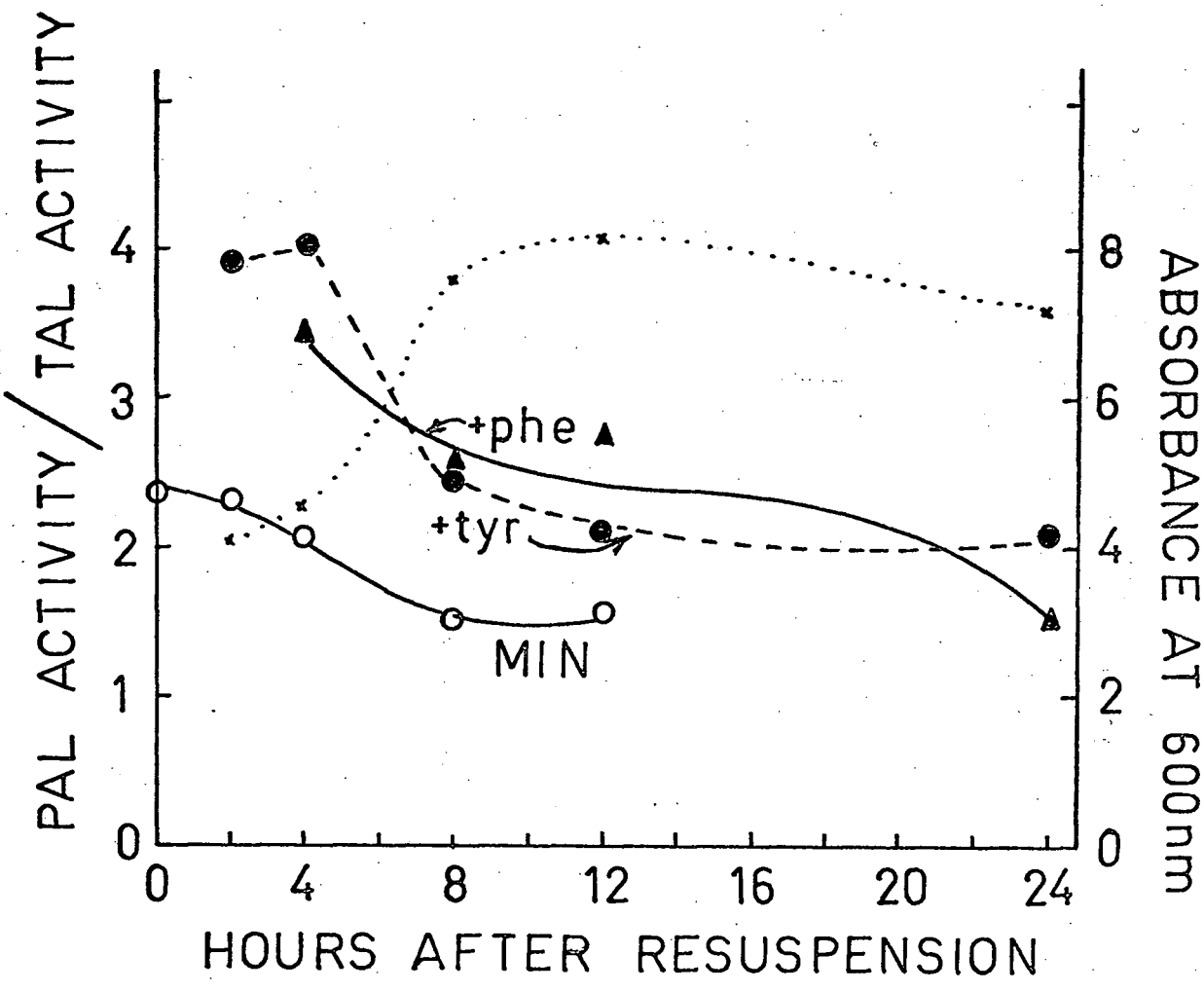


FIGURE 19

Growth and change in ratio of PAL activity to TAL activity of resuspended cells. (absorbance ··x··; cells in minimal medium —o— ; minimal medium plus tyrosine – –o– –; minimal medium plus phenylalanine —△—)

the presence of phenylalanine. Both species were grown on malt medium for 48 hours, then were resuspended in .01% phenylalanine growth medium. In both cultures, a sharp increase in the amount of enzyme was observed four hours after resuspension, followed by a slow decline. In R. texensis, the level of PAL doubled; in R. glutinis, the level increased ten-fold.

Sporobolomyces roseus behaves like R. texensis in that both enzymes are produced at low levels even when the cells are grown in glucose. Unlike Rhodotorula, however, Sporobolomyces shows no immediate burst of enzyme production when supplied with phenylalanine. Since Ogata et al supply no growth data for their resuspended cells, there is no information as to the growth stage of the cells during this peak of enzyme activity. The increase in activity of these cells might occur during logarithmic phase, as in Sporobolomyces roseus, rather than in immediate response to the presence of phenylalanine.

The work on Rhodotorula (Ogata et al, 1967a) did not include any attempts to repress enzyme activity. It has been shown in Sporobolomyces that PAL and TAL activities are effectivity inhibited by cinnamic acid and by p-coumaric acid. Since the ether extracts of these cinnamic acid-supplied cells show that there is cinnamic acid in the cells, it is possible that the cinnamic acid is merely inhibiting the PAL and TAL present in the acetone powder. The low concentration of cinnamic acid in the buffer extract of the acetone powder makes it unlikely that inhibition of activity can fully account for the low PAL levels. Furthermore, there was no p-coumaric acid found in the p-coumaric acid-supplied cells, so product inhibition cannot be the complete answer in the case of TAL either. A more likely explanation is that the production of the enzymes has been repressed by the presence of the reaction products.

Feedback repression (the repression of the synthesis of an enzyme by the presence of high levels of reaction product) prevents the formation of unnecessarily high levels of that product. The phenomenon has been observed to operate in the synthesis of amino acids, and prevents the formation of more amino acid than is being incorporated into protein. The degradation of the aromatic amino acids, too, is under this sort of control. Zucker has shown in potato tubers that the presence of cinnamate appears to reduce that rate of PAL synthesis by acting as a feedback repressor (Zucker, 1965).

The enzymes PAL and TAL resemble inducible enzymes in several ways, since production is stimulated by substrate and inhibited by glucose. Yet even in the absence of inducer, enzyme production continues at a low level. In this respect, Sporobolomyces roseus UBC 901 appears similar to paraconstitutive mutants of E. coli (Jacob and Monod, 1961). In the absence of inducer, these mutants product enzymes which are normally inducible at a level greater than zero but less than the wild-type maximum. In the presence of inducer, the enzyme content increases in both wild and mutant strains. It would be very interesting to see if other isolates of Sporobolomyces roseus also produce PAL and TAL when grown on glucose, or if they behave in a manner analogous to a wild strain.

In nature, Sporobolomyces grows on the surface of leaves and fruit of higher plants. It seems likely that the concentration of p-coumaric and cinnamic acids in the substratum would be relatively high at all times, and that the ammonia lyases would remain repressed. During balanced growth, phenylalanine and tyrosine would not be produced by the plant in large amounts; thus the substrate for the enzymes would be present only in low concentrations. Lee and Arnonoff (1967) have shown that one result of borate deficiency in higher plants is a greater use of the pentose phosphate pathway and

subsequent increase in shikimic acid precursors. The result is an increase in the production of phenolic acids, and probably also phenylalanine and tyrosine. Perhaps it is in such cases of unbalanced growth of the host organism resulting in high levels of production of both substrate and products of the ammonia lyases that the control system over these enzymes is effective.

Since PAL and TAL production can be repressed by p-coumaric or cinnamic acid, it would be interesting to test repressed cells for alternate pathways of degradation of the aromatic amino acids. Perhaps even Sporobolomyces roseus can form phenylpyruvic acid. It would also be of interest to see if benzoic acid and p-hydroxybenzoic acid are also inhibitors of PAL and TAL.

Further purification should also be carried out. The present partial purification was carried out on buffer extracts of cells ground by hand with alumina. Near the end of the study, it was found that extracts of acetone powders prepared from frozen cells provided very active enzyme preparations. Future purification attempts would probably be more fruitful if easily prepared acetone powder extracts, which are free from much cellular material such as carotenoids, were to be used as the starting material.

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