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Tryptophanase Jnduction in *Pasteurella multocida*

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Introduction

Numerous reports have been accumulated concerning inducible formation of enzymes by bacteria of which nutritional requirement is not exact. Details of mechanism underlying enzyme induction have steadily been clarified. On the other hand reports about that by nutritionally exact bacteria have been extremely few in number.

It is of much interest to examine whether exact organisms requiring vitamins, protein digest or beef extract are able to produce enzyme protein soly in the presence of amino acids and an inducer or necessitate nutrients such as beef extract, protein digest or vitamins in addition to them. If these organisms require such nutrients, the question arises what are functions played by the nutrients in the course of enzyme synthesis.

Pasteurella multocida, which is the causative organism of fowl cholera, generally can not grow in medium composing of amino acids and glucose and requires a suitable amount of vitamins (thiamin, nicotinamide or pantotenate), protein digests or beef extract in addition to them (3, unpublished data). Experimental results regarding tryptophanase induction by *Pasteurella multocida* obtained suggest that this reaction reflects induced synthesis of enzyme protein, and indicate that beef extract is necessary for the reaction. The present report is a description of experimental results concerning these problems.

Materials and Methods

Bacterial strain used. *Pasteurella multocida*, strain TS–8.

Buffer. M/15 phosphate buffer, pH 7.0.

The condition of enzyme induction. The cells grown by shaking in broth at 37°C overnight were washed once with buffer, and resuspended in buffer in the concentration of 3×10^8 cells per ml. Aliquots of 0.3 ml of the cell suspension were put in tubes, otherwise noted. 0.05 ml of 10% casamino acid solution was added to each tube as nitrogen source. 0.005 M L-tryptophan, 0.01 M galactose, 0.01 M mannitol, 0.01 M sucrose or 0.01 M potassium nitrate was added to tubes in the case of TPase, galactose fermentation system, mannitol fermentation system, sucrose fermentation system or nitrate reductase, respectively. Total volume was 0.5 ml. The reaction mixtures were incubated at 37°C for 180 minutes without shaking.

Preincubation for starvation. The cells grown in broth were washed twice with buffer and resuspended in the buffer containing 1% casamino acid in the concentration of 1.8×10^7 cells per ml, followed by shaking at 37°C for 180 minutes.

Enzyme assay. TPase was assayed as follows: 0.5 ml of reaction mixtures was centrifuged and induced cells were washed once with buffer, followed by the resuspension in 0.25 ml of buffer. These suspensions were incubated in the addition of 0.005 M L-tryp-

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tophan and 10 μg of chloramphenicol at 37°C for appropriate times. Using the procedures described in Methods in enzymology[2] amounts of indole produced were determined. The TPase unit is defined as that amount of enzyme required to form 1 ug of indole per 10-minute assay. Sugar fermentation systems were assayed as follows: after centrifugation and washing of reaction mixture, the cells were resuspended in 0.5 ml of buffer and incubated at 37°C for 15 hr with the addition of 0.01 M galactose, mannitol or sucrose corresponding to each sugar fermentation system and 20 ug of chloramphenicol. The amounts of 0.1% sodium hydroxide requiring for neutralization of produced acids were shwon at Table 1 as a measure of enzyme activities. Nitrate reductase was assayed owing to the procedures described in Methods in enzymology[7], with exception that one unit was defined as that amount of enzyme which results in the production of 0.1 ug per 10-minute assay.

Table 1. The increase in activities of various enzymes or enzyme systems dependent upon the additions of substrate and amino acies

Additions	Enzymes or enzyme systems	Enzymic activities/0.5 ml of induction mixture			
		Substrate			
		Absence		Presence	
		0′	180′	0′	180′
LAH	TPase	13	47.5	18.9	71.2
	Galactose fer.	0	0.02	0	0.08
	Mannitol fer.	0	0.01	0	0.06
	Sucrose fer.	0	0.01	0	0.015
	Nitrate red.	9	9	9	9.5
Vitamin mixture	TPase	13	13	18.6	70.2
	Galactose fer.	0	0	0	0.03
	Mannitol fer.	0	0	0	0.07
	Sucrose fer.	0	0	0	0.025
	Nitrate red.	9	8	8.5	8.5
None	TPase	12.4	12.7	19.2	71.6
	Galactose fer.	0	0	0	0.05
	Mannitol fer.	0	0	0	0.06
	Sucrose fer.	0	0	0	0.015
	Nitrate red.	8.5	9.5	9	8.5
-CA	TPase			13.8	11.8
	Galactose fer.	0	0	0	0.02
	Mannitol fer.	0	0	0	0
	Sucrose fer.	0	0	0	0

Abbreviations: TPase=tryptophanase; fer.=fermentation system; LAH=lactalbumin hydrolysate (enzymatically); CA=casamino acid. Final concentrations of added components were as follows: CA 1%; LAH 0.2%; thiamine 10 μg per ml; pantotenate ca. 1 μg per ml; nicotinamide 10 μg per ml; hematin 5 μg per ml. Basal medium for enzyme induction was composed of a substrate (an inducer), 1% CA and buffer. The reaction mixtures were incubated at 37°C for 180 minutes.

Preparation of extracts from induced and noninduced cells. After washing of cells grown in 90 ml of broth they were induced for TPase for 180 minutes and resuspended in 5 ml of buffer and subjected to disruption with French pressure cell (at 400 kg per cm^2). From the homogenate extract was obtained with centrifugation of three times at 1,8000xg for twenty

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minutes. From cells grown in broth noninduced cells extract was prepared with the same procedures as in induced cells.

Results

First of all the inducibility of increase in enzyme activity was examined on several enzymes or enzyme systems. These results are given at Table 1. Enzyme systems participating in sugar fermentation increased in activity dependent upon the presence of substrate and amino acids; these enzyme systems are presumably thought to be inducibly formed. However, these enzyme systems, because of low activity, are somewhat inadequate for the research of enzyme induction in this bacteria. Nitrate reductase was found to be a constitutive enzyme although it is inducible one in *Escherichia coli*. Contrary to these enzymes TPase was an inducible enzyme having a considerably high activity and seems to be satisfactory for the study of enzyme induction. The above cause let me direct toward the study of TPase induction.

Figure 1 shows the time course of TPase. In spite of resting cells lag phase was not

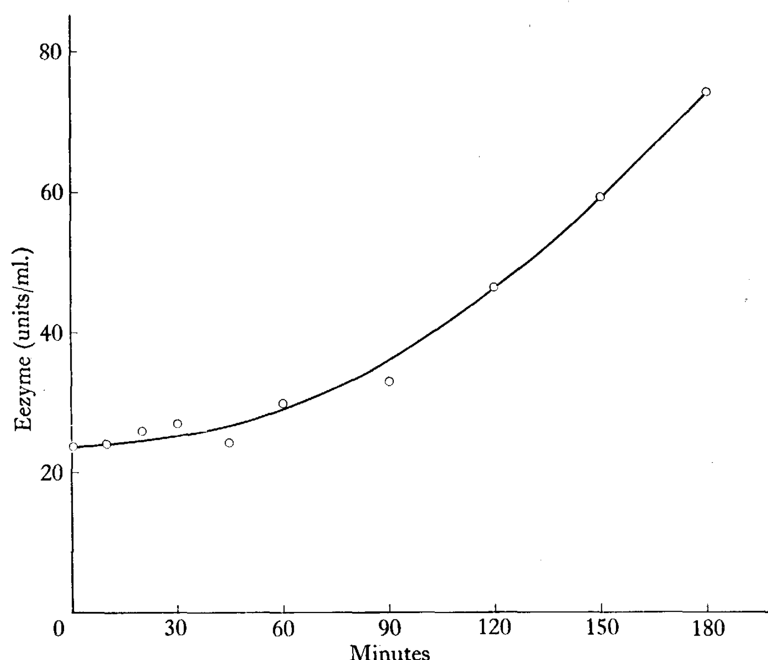

Fig. 1. Time course of TPase induction.

observed such as found in β-galactosidase formation in *Escherichia coli*. Since some amount of tryptophan contained in broth elicited TPase induction during the overnight growth in broth, broth-grown cells had already considerable amount of TPase. The maximum peak found in TPase induction was approximate to 35°C (Fig. 2) roughly identical to that in growth. At pH 8.0 the enzyme was induced most actively, whereas at a range of actidity or alcali the enzyme induction occurred at a low level or did not occur at all (Fig. 3). Although the rate in TPase induction was invariable at a concentration of more than 2.4×10^8 cells per ml, it lowered markedly at a thin concentration of cell (Fig. 4).

In the next place the effects of various factors on TPase induction were examined (Table 2). The depletion of amino acids from induction medium resulted in the lack of enzyme induction; TPase induction was completely dependent upon the external supply of amino acids in spite of cells grown in broth. Such a phenomenon is never seen in *Escherichia*

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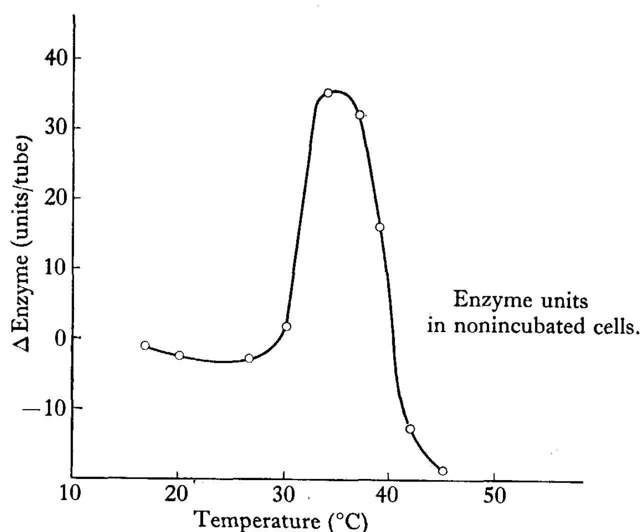

Fig. 2. Effect of temperature on TPase induction.

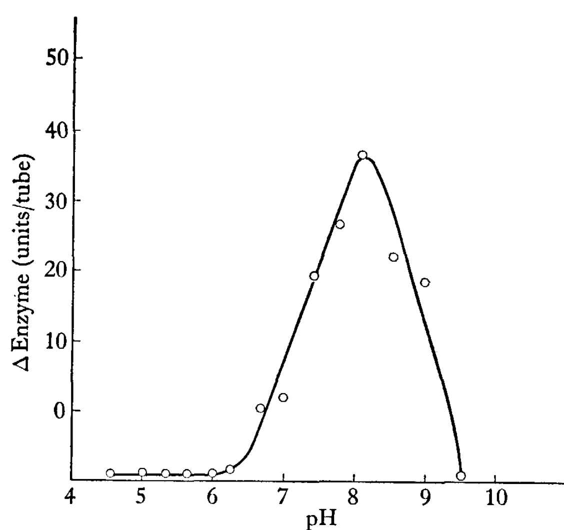

Fig. 3. Effect of hydrogen concentration on TPase induction. Buffers indicating a given hydrogen concentration were prepared by the mixture of appropriate volume ratio of M/15 potassium phosphate (monobasic) and M/15 sodium phosphate (dibasic). Buffers indicating alcali were adjusted to a given hydrogen concentration by the addition of 1% sodium hydroxide. After washing of cells once with each buffer the cells were suspended with the buffer and induced.

Table 2. Effects of various factors on TPase induction

Conditions of incubation	TPase activities (units/tube)
Basal medium	45.9
〃 −CA	11.7
〃 +CM, 30 μg per ml	4.8
〃 +Glucose, 0.05 M	21.0
〃 +Beef extract concentrated 3-fold	31.2
〃 +Beef extract	48.3
〃 +Beef extract diluted 10-fold	50.5
〃 +LAH, 5%	41.5
〃 +LAH, 1%	56.0
〃 +LAH, 0.2%	55.2
〃 +Vitamin mixture	51.3
(nonincubated)	12.9

Abbreviation: CM=chloramphenicol.
Notes described in Table 1 are also applied to this table. Concentration of beef extract is identical to that in broth medium used for bacterial culture.

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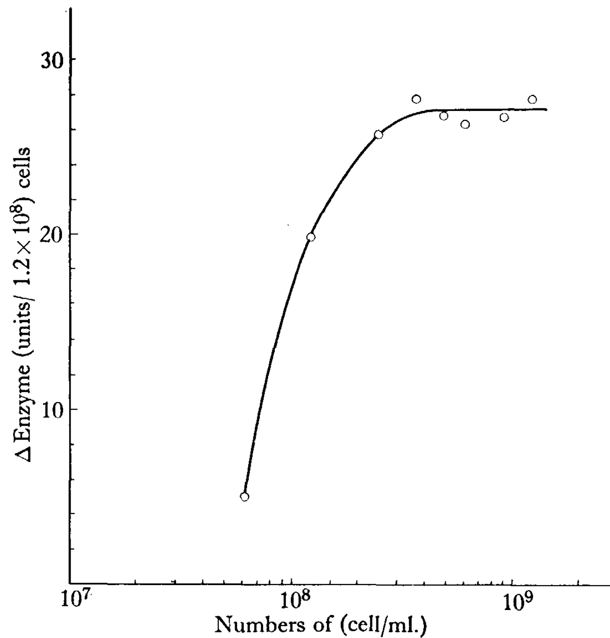

Fig. 4. Effect of concentration of cell on TPase induction.

coli. 30μg per ml of chloramphenicol prevented completely the induction, and 0.05 M of glucose inhibited strongly the reaction. Beef extract, lactalbumin hydrolysate (enzymatically. purchased from Nutritional Biochemicals Corporation) or vitamin mixture, which are all essential nutrients for the growth, promoted TPase induction only a little. Sometimes they did not activate the reaction. The addition of beef extract or protein digest in high concentrations inhibited TPase induction on the contrary. Further experiments on each factor were made. Chloramphenicol prevented completely at 20 ug per ml, and in higher concentrations of the drug the enzyme content in the incubated cells decreased below that in the noninduced cells (Fig. 5). Glucose stimulated significantly the induction at approximate 0.004M, whereas in higher concentrations prevented completely. This phenomenon is probably the reflection of promotion of enzyme repression by glucose (Fig. 6).

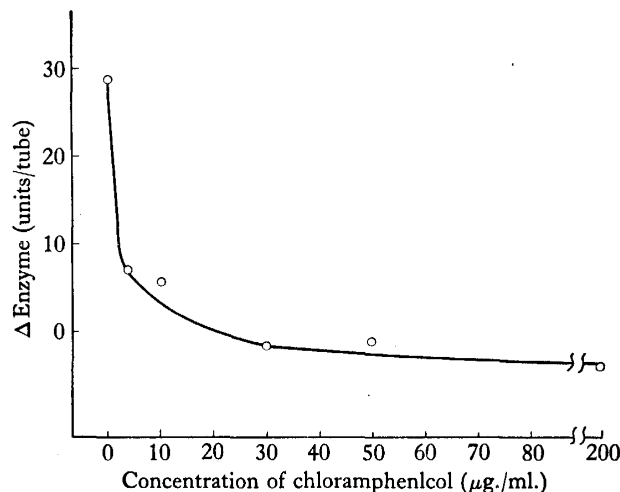

Fig. 5. Effect of chloramphenicol on TPase induction.

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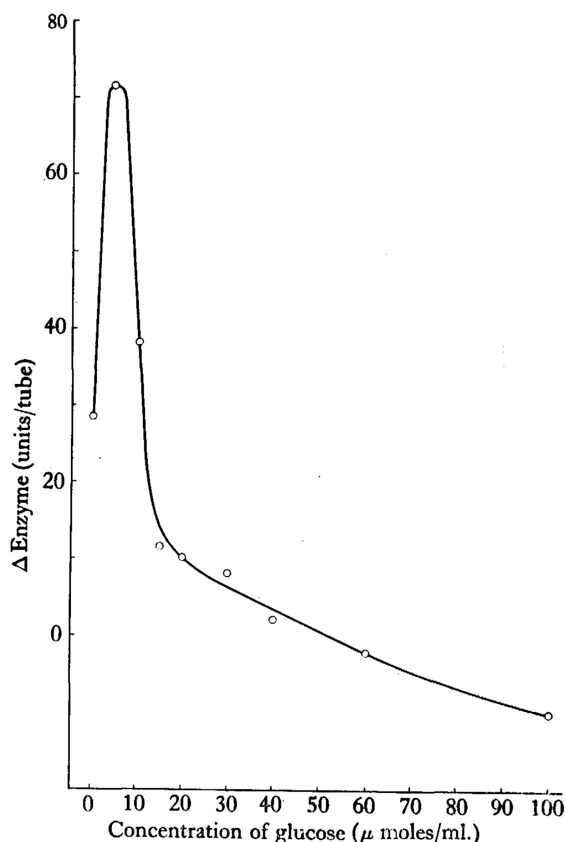

Fig. 6. Effect of glucose on TPase induction.

To ascertain whether TPase induction is based upon the protein synthesis, amino acid- or purine-analogue was added to the induction mixtures. Table 3 indicates that p-fluorophenylalanine inhibited the induction and the inhibition was reversed by further addition of phenylalanine. Similarly, 8-azaguanine inhibited the induction and the further addition of RNA or RNA digest reversed the inhibition. These findings indicate the participation of amino acid or purine. These facts suggest that TPase induction is based upon protein synthesis. The addition of these analogues occasionally decreased enzyme content far below that in noninduced cells. Data on effect of 2,4-dinitrophenol is given in Table 4. 0.00005 M of 2,4-dinitrophenol prevented completely TPase induction. Remarkable decrease of the enzyme content below initial level was observed in cells incubated under the presence of high concentration of this drug.

In the next place TPase activity in extract prepared from induced cells was compared with that from noninduced cells. As shown at Table 5, TPase activity in extract from induced cells was higher surely than that from noninduced cells.

All findings shown hitherto are in accordance with the interpretation that the increase in TPase activity dependent upon the addition of substrate and amino acids is the reflection of induced synthesis of enzyme protein.

In order to clarify the effects of nutrients such as beef extract, protein digest and vitamins, the starvation of cells grown in broth was attempted. This object was attained by shaking cells in the presence of casamino acid under the concentration of 1.8×10^7 cells per ml at 37°C for 180 minutes. The same procedures under the concentration of 1.2×10^8 cells per ml did not cause a starvation of cells. Data regarding TPase induction by cells subjected to starvation from broth nutrients are summarized in Table 6. In the sole addition of casamino

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Table 3. The inhibitory effects of amino acid- or purine-analogue on TPase induction

Conditions of incubation	TPase adtivities (units/ml)	
	0.1 M phe	
	Absence	Presence
Basal medium	39.8	
" +0.016 M p-FPA	5.0	7.5
" +0.008 M p-FPA	18.6	39.8
" +0.0024 M p-FPA	16.6	39.2
" +0.0008 M p-FPA	43.5	39.0
" −CA+*amino acids	9.6	
" −CA+amino acids+0.008 M p-FPA	1.1	8.5
" −CA+amino acids+0.0024 M p-FPA	2.0	4.7
" −CA+amino acids+0.0008 M p-FPA	1.5	2.3
(nonincubated)	6.0	
	1% RNA	
	Absence	Presence
Basal medium	39.8	
" +0.0066 M 8-aza	2.6	16.3
" +0.00198 M 8-aza	10.1	14.9
" +0.00066 M 8-aza	29.4	26.9
(nonincubated)	6.0	
	**RNA digest	
	Absence	Presence
Basal medium	57.0	
" +0.0066 M 8-aza	0	8.0
" +0.00198 M 8-aza	7.6	42.0
(nonincubated)	12.0	

Abbreviations: p-FPA=p-fluorophenylalanine; 8-aza=8-azaguanine; phe=DL-phenylalanine; RNA=ribonucleic acid. Enzyme units per ml of enzyme asay mixture are described.
* Mixture of eighteen amino acids omitting phenylalanine. 200 μg per ml of each amino acid was added.
** 5% yeast RNA solution was hydrolysed with RNase of 10 μg per ml for 220 minutes at 37°C and 0.1 ml of the RNA digest was added to 0.4 ml of reaction mixture.

Table 4. Effect of 2,4-dinitrophenol on TPase induction

Concentration of 2,4-dinitrophenol	TPase activities (units/bute)
0.008 M	0
0.005 M	1.0
0.0005 M	2.2
0.00005 M	15.6
0	38.2
(nonincubated)	18.8

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Table 5. Comparison of TPase activity in extract from cells induced and noninduced

Sort of extracts	TPase activities
Extract from induced cells	36.3
Extract from noninduced cells	10.0

Table 6. TPase induction by starved cells

Additions	TPase activities (units/tube)
None	23.8
Beef extract	63.2
Beef extract diluted 2-fold	59.3
Beef extract diluted 4-fold	57.6
Beef extract diluted 8-fold	52.5
Beef extract diluted 16-fold	40.7
0.05 M glucose	49.9
0.025 M glucose	46.3
0.0125 M glucose	47.2
0.00625 M glucose	45.7
0.00375 M glucose	52.6
0.00156 M glucose	50.5
Beef extract+0.05 M glucose	47.3
0.2% LAH	22.0
0.2% LAH+0.00375 M glucose	44.4
Tryptic digest of casein (0.1%)+0.00375 M glucose	48.9
Tryptic digest of lipase (0.1%)+0.00375 M glucose	44.7
Peptic digest of lipase (0.1%)+0.00375 M glucose	44.2
Vitamin mixture	17.4
Vitamin mixture+0.00375 M glucose	51.1
(nonincubated)	40.0

The cells grown in broth were shaken in the presence of 1% casamino acid at 37°C for 180 minutes under the concentration of 1.8×10^7 cells per ml and induced with the same procedures under the addition of indicated substances.
Protein digests were prepared as follows: 1.2% protein solutions were incubated in the presence of 0.1% trypsin (in M/15 phosphate buffer, pH 7.0) or 0.1% pepsin (in M/10 sodium citrate-HCl buffer, pH 2.0) at 37°C for 180 minutes.

acid TPase was not induced at all. Besides that enzyme level lowered markedly. Only beef extract exhibited a relatively clear stimulation in proportion to its concentration. It is of interest that glucose exerted some promotion independent of its concentration. However, experimental cases not showing an active effect with glucose were found. Glucose decreased the rate of stimulation by beef extract. The addition of protein digest or vitamin mixture resulted in a remarkable decrease in enzyme activity, as seen in the induction in the sole addition of casamino acid. Such starved cells preserved fully growth activity in broth.

A stimulation by broth was also observed in TPase induction with cells starved by shaking at 37°C for 180 minutes in semi- synthetic medium, which permits the growth of strain TS-8 (Table 7). In contrast to the above cells, a small activation by protein digest was found in these cells.

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Table 7. TPase induction by cells starved by shaking in a semi-synthetic medium

Additions	TPase activities (units tube)
None	47.9
0.2% LAH	51.0
Beef extract	79.3
(nonincubated)	45.0

Semi-synthetic medium was composed of M/15 phosphate buffer (pH 7.0), 1% casamino acid, 0.5% glucose, 0.02% magnesium sulfate and vitamin mixture.
Conditions for starvation was the same as that described in Table 6 with exception that a semi-synthetic medium was used for starvation in place of buffer containing 1% casamino acid.

Pasteurella multocida is exact bacteria in nutrition. However, such a mutation occurred by chance in strain TS-8 used in this study that this strain became not to necessitate these nutrients. From such a mutant starved cells were prepared owing to the same procedures and the same experiment was performed. As shown at Table 8, amino acids provoked of

Table 8. TPase induction by starved cells of mutant of strain TS-8

Additions	TPase activities (units/tube)
None	50.1
0.05 M glucose	20.3
0.004 M glucose	32.1
0.004 M glucose+0.2% LAH	25.8
0.004M glucose+vitamin mixture	43.9
0.2% LAH	71.5
Beef extract	71.0
(nonincubated)	23.3

TPase induction and glucose elicited a repression in both lower and higher concentration. Beef extract and protein digest exhibited a stimulation. In view of these findings it is thought that properties demonstrated in Table 6 may possibly be characteristic of nutritionally severe bacteria.

Discussion

A series of experiments about TPase induction by cells grown in broth of *Pasteurella multocida* has been carried out. These experiments suggest strongly that the reaction is based upon induced synthesis of the enzyme protein. The effect of beef extract on enzyme induction, which was not obvious in cells grown in broth, became evident considerably by the starvation of broth-grown cells. It is quite inexplicable why TPase formation by the starved cells is not repressed with glucose even in higher concentration but conversely promoted slightly. The effects of protein digest and vitamins on enzyme formation were slight, sometimes ambiguous in contrast to the absolute requirement for the proliferation of these nutrients. It seems to be somewhat paradoxical that these slight effects were observed only in enriched cells whereas not found at all in satrved cells. Subsquent experiments indicate that cells grown in a semi-synthetic medium composed of casamino acie, vitamins and in organi csalts possessed an ability to induce TPase. One explanation for these situations is as follows: since broth-grown cells preserving intracellular nutrients abosrbed from broth medium have

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ability to produce the enzyme, additional effects of protein digest and vitamins can exert on the enzyme induction. As the process of exhaustion of nutrients proceed, any variation relevant to the properties of enzyme formation may occur inside cells. This variation can be restored by beef extract, but not by protein digest or vitamins. However, this explanation may be unable to testify the effects of glucose on TPase induction by starved cells. It was observed in author's experiments that there may be differences between the effects of beef extract and protein digest on the growth of *Pasteurella multociea* (unpublished data). It is of interest that a difference between these effects was found also in TPase induction.

Maretzki and Mallette[6] reported a stimulation by peptides of lysine decarboxylase formation, and synthesis of streptococcal proteins was found to be dependent upon the addition of peptides. From experiments with *Lactobacillus mesenteroides* Kihara and Shell[4,5] thought that peptides promote a bacterial growth by alleviating the competitive inhibition of individual amino acids in the permeation into cells. Various observation, however, about effects of peptides on the growth of *Pasteurella multocida* suggest that their explanation may not necessarily be fully satisfactory (unpublished data).

It is of interest that the incubations for enzyme induction in unfavoarble conditions caused a decrease in TPase activity below initial enzyme level, although a mechanism of this phenomenon is quite inexplicable. The decrease in TPase activity was caused by incubation in the presence of amino acids at 37°C under the conditions where protein synthesis was blocked, but incubation in the absence of amino acids or in a low temperature did not almost decrease the enzyme activity. The situation found in the incubation in the additions of eighteen amino acids and p-fluorophenylalanine of 0.0024 M or 0.0008 M seems to be complicated (Table 3); the further addition of phenylalanine of 0.1 M apparently reversed the inhibition by p-fluorophenylalanine, nevertheless the enzyme level in cells reached after the incubation in the further addition of phenylalanine was lower than that in nonincubated cells. These problems will be studied in the near future.

Summary

An increase in tryptophanase activity in various conditions was examined in *Pasteurella multocida.* These experiments suggest strongly that an increase in tryptophanase activity dependent on the supply of an inducer and amino acids is based upon induced synthesis of the enzyme protein.

Effects of beef extract, protein digest or vitamin mixture on tryptophanase induction by cells grown in broth were only slight. On the other hand the situation in tryptophanase induction by cells subjected to starvation was different from that by cells grown in broth in several points; starved cells were not capable of forming the enzyme in the presence of an inducer and amino acids, but could produce the enzyme completely depending on the addition of beef extract. Also, glucose possessed an ability to provoke of enzyme induction in such starved cells, although to a small extent. In contrast protein digest or vitamin mixture had not such an ability in starved cells.

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References

1) Fox, E.N. 1961. Peptide requirements for the synthesis of streptococcal proteins. *J. Biol. Chem.,* **236**: 166–171.

2) Gunsall, I.C., Galeener, C.C. and Stamer, J.R. 1955. Tryptophan cleavage, p. 238–242. In Colowick, S.P. and Kaplan, N.O. (eds.), Methods in enzymology. Vol. 2. Academic Press Inc., Publishers, New York.

3) Jordan, R.M.M. 1952. The nutrition of *Pasteurella multocida.* II. The formation of hydrogen peroxide in a chemically-defined medium. *Brit. J. exper. Pathol.* **33**: 36–45.

4) Kihara, H. and Snell, E.E. 1960. Peptides and bacterial growth. VIII. The nature of strepogenin. *J. Biol. Chem.,* **235**: 1409–1414.

5) Kihara, H. and Snell, E.E. 1960. Peptides and bacterial growth. IX. Release of double inhibitions with single peptides. *J. Biol. Chem.,* **235**: 1415–1418.

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