we have a similar situation with Bence-Jones and myeloma proteins, particularly since Stevenson (1960) has reported that Bence-Jones-like proteins may be present in normal human urine.

SUMMARY

1. The serum myeloma protein and urinary Bence-Jones protein of mice carrying the MPC-2 plasma-cell neoplasm have been purified by chromatography on ion-exchange diethylaminoethyl-cellulose and characterized.

2. The neoplastic plasma cells were found to form both proteins. Biosynthesis was studied by incubating sliced tumour tissue *in vitro* and following the incorporation of [¹⁴C]amino acids into the Bence-Jones protein and myeloma protein isolated by chromatography on diethylaminoethyl-cellulose and by precipitation with rabbit antiserum. The time course of incorporation of radioactive amino acid into the two proteins is consistent with the view that Bence-Jones and myeloma proteins were formed independently by the neoplastic plasma cells and that neither protein was a precursor or breakdown product of the other. Approximately two molecules of Bence-Jones protein were formed for each molecule of myeloma protein.

3. The pool of extracellular plus intracellular protein associated with MPC-2 tumour tissue was determined by isotope-dilution techniques employing ¹³¹I-labelled Bence-Jones and ¹³¹I-labelled myeloma protein. About 3.6 mg. of myeloma protein and 0.4 mg. of Bence-Jones protein were found in 1 g. of tumour tissue (wet weight). This difference is probably caused by the rapid clearance of Bence-Jones protein from the plasma and extracellular fluid by excretion through the urinary tract.

We are most grateful to Dr M. Potter, National Insti-

tutes of Health, Bethesda, for making this investigation possible by sending us the MPC-2 tumour line and breeding pairs of his BA1b/c mouse strain. We are also most indebted to Dr Peter Charlwood for measurements of S_{20} and molecular weights of the protein samples, Mrs Marion Perryman for her excellent assistance, and to Dr J. H. Humphrey for his advice.

REFERENCES

- Aronsson, T. & Grönwall, A. (1958). *Scand. J. clin. Lab. Invest.* **10**, 348.
 Askonas, B. A. (1961). *Biochem. J.* **79**, 33.
 Eagle, H. (1955). *J. biol. Chem.* **214**, 839.
 Fahey, J. L. & Horbett, A. P. (1959). *J. biol. Chem.* **234**, 2645.
 Fahey, J. L. & Potter, M. (1959). *Nature, Lond.*, **181**, 1419.
 Freund, J. & McDermott, K. (1942). *Proc. Soc. exp. Biol., N.Y.*, **49**, 548.
 Hanks, J. H. (1948). *J. cell. comp. Physiol.* **31**, 235.
 Korngold, L. & Lipary, R. (1956). *Cancer*, **9**, 262.
 McFarlane, A. S. (1958). *Nature, Lond.*, **182**, 53.
 Meyer, F. (1957). *Fed. Proc.* **16**, 222.
 Nathans, D., Fahey, J. L. & Potter, M. (1958). *J. exp. Med.* **108**, 121.
 Osserman, E. F., Graff, A., Marshall, M., Lawlor, D. & Graff, S. (1957). *J. clin. Invest.* **36**, 352.
 Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
 Potter, M., Fahey, J. L. & Pilgrim, H. I. (1957). *Proc. Soc. exp. Biol., N.Y.*, **94**, 327.
 Putnam, F. W. (1960). In *The Plasma Proteins*, vol. 2, p. 346. Ed. by Putnam, F. W. New York: Academic Press Inc.
 Putnam, F. W. & Hardy, S. (1955). *J. biol. Chem.* **212**, 361.
 Putnam, F. W. & Miyake, A. (1958). *J. biol. Chem.* **231**, 657.
 Robbins, J., Wolff, J. & Rall, J. E. (1959). *Endocrinology*, **64**, 12, 37.
 Smithies, O. (1955). *Biochem. J.* **61**, 629.
 Sober, H. A., Gutter, F. J., Wyckoff, M. M. & Peterson, E. A. (1956). *J. Amer. chem. Soc.* **78**, 756.
 Stevenson, G. T. (1960). *J. clin. Invest.* **39**, 1192.

Biochem. J. (1961) **80**, 268

Induced Formation of Leucine Decarboxylase in *Proteus vulgaris*

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(Received 2 February 1961)

The formation of many microbial enzymes is directly stimulated or 'induced' by the presence of the corresponding substrate or some related compound. Inducers must presumably combine with

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some specific receptor within the cell in order to exert their effects. The earliest and simplest suggestion was that this receptor was the enzyme itself (Yudkin, 1938). If small amounts of an enzyme exist in equilibrium with large amounts of its precursors a substrate or competitive inhibitor

might, by combining with the enzyme, stimulate formation of the latter by a direct mass-action effect.

Monod and his co-workers showed that this simple theory was not tenable for β -galactosidase induction in *Escherichia coli* (Monod, Cohen-Bazire & Cohn, 1951; Cohn & Monod, 1953). Certain substrates of that enzyme failed to act as inducers, whereas some inducers were considered incapable of combination with the enzyme, being neither substrates nor inhibitors. They suggested that inducers combine with some receptor other than the enzyme and that the resulting complex has a catalytic role in enzyme formation. This 'catalyst theory' of induction could account for the behaviour of other adaptive enzyme systems also.

Proteus possesses an amino acid decarboxylase which has been studied in this Laboratory (Ekladius, King & Sutton, 1957). Leucine, valine, norvaline, isoleucine and α -amino-*n*-butyrate are attacked, apparently by a single enzyme. This will be referred to as 'leucine decarboxylase' since leucine is the amino acid most readily attacked. Cells grown on a simple synthetic medium contain little of the enzyme unless leucine or valine is included in the medium. This suggested that the enzyme was adaptive, like the amino acid decarboxylases described by Gale (1940), and that leucine and valine function as inducers.

This system offers certain advantages as material for investigations on enzyme induction. The several known substrates and competitive inhibitors provide a range of substances whose potentialities and activities as inducers could be compared. The Michaelis constants for the substrates are relatively high, allowing exposure of the organism to a potential inducer at concentrations high enough to avoid complete metabolic destruction over a reasonable experimental period, yet well below that required to saturate the enzyme. In a number of cases induction was studied over a range of inducer concentrations, and the affinity of the inducer-accepting centre determined by application of orthodox kinetic methods. This provided the necessary data for deciding whether combination of the enzyme with its substrate plays any major role in the induction process.

MATERIALS AND METHODS

Organism and medium. A laboratory strain of *Proteus vulgaris* was used. For experiments on induced enzyme formation a stock culture was used which, 2 years previously, had been transferred from broth and maintained on synthetic medium.

The synthetic medium contained: 70% syrupy sodium lactate (British Drug Houses Ltd.), 0.8% (v/v); nicotin-

amide, 5 μ g./ml.; NaCl, 0.5%; $(\text{NH}_4)_2\text{SO}_4$, 0.8%; KH_2PO_4 , 0.2%; Na_2HPO_4 , 0.2%. The medium was made up in tap water and autoclaved for 30 min. at 5 lb./in.² The pH after autoclaving was 6.8.

Enzyme preparations. For studies on enzyme induction the organism was grown at 37° in Roux bottles containing 100 ml. of the synthetic medium. When the bacterial density reached a value between 0.3 and 0.8 mg. dry wt. of bacteria/ml., the cultures were pooled and redistributed in 80 ml. portions in Roux bottles. An inducer or other compound under investigation, or both, was added, dissolved in 20 ml. of 0.1 M- KH_2PO_4 - Na_2HPO_4 buffer, pH 7.0. Incubation at 37° was continued for 3 hr. unless otherwise stated. The cells were then harvested by centrifuging for 20 min. at 2900 g and washed twice on the centrifuge with 1% NaCl (80 ml. each time). They were then suspended in 0.1 M-phosphate buffer, pH 6.0, to a density of about 10 mg. dry wt./ml., and the decarboxylase activity was estimated. Cell densities were determined with the EEL portable colorimeter (Evans Electroelenium Ltd., Harlow, Essex), which had been calibrated by direct determinations of dry wt.

For studies on enzyme action cells were grown on nutrient broth and washed suspensions were prepared as previously described (Ekladius *et al.* 1957). Cell-free extracts were obtained by shaking with glass beads (Ballotini no. 12; King & Alexander, 1948) or by crushing in the Hughes press at -20° without abrasive (Hughes, 1951). The extracts were freeze-dried and stored at -10°. Before use, a weighed amount of freeze-dried material was suspended in buffer, pH 6.0, and centrifuged for 20 min. at 2400 g, and the sediment discarded.

Assay of decarboxylase activity. Warburg manometers filled with N_2 were used at 37°. The main compartment of the Warburg flasks contained the decarboxylase preparation (10-25 mg. dry wt. of cells or an equivalent amount of cell-free extract) and the coenzyme, pyridoxal phosphate (15 μ g.). The substrate was placed in a side arm, and any compound to be tested as an inhibitor was placed in a second side arm. L-Leucine (175 μ moles) was used as substrate for cell-free enzyme preparations; with intact cells however DL-valine (350 μ moles) was preferred as, under these conditions, it gave linear CO_2 output more consistently over long periods.

All these substances were in 0.1 M- KH_2PO_4 - Na_2HPO_4 buffer, pH 6.0, and sufficient of this buffer was added to the main compartment to bring the final volume to 3.0 ml. Decarboxylation was followed for 40 min. after tipping in the substrate.

In one experiment, when only low enzyme activities were expected, decarboxylation was measured by the isotopic method of Gale (1957). Conway units contained, in the outer compartment, the enzyme preparation (1.0 ml.), pyridoxal phosphate (10 μ g. in 0.2 ml.) and DL-[1-¹⁴C]-valine (0.4 ml. containing 0.2 m-mole of specific activity 3 μ Ci/m-mole). In the inner compartment was a planchet containing baryta as described by Gale. The units were filled with N_2 to prevent oxidation of the substrate by the highly active amino acid oxidase present in *Proteus*. Decarboxylation was allowed to proceed for 30 min.

Units. Enzyme activity is expressed as $Q_{\text{CO}_2}^{\text{N}}$ (μ l. of CO_2 /mg. dry wt./hr.). Under the conditions used, there was no measurable gas evolution unless a substrate was added.

Enzyme induction during the standard 3 hr. period is expressed as the differential rate of enzyme formation, i.e. the increase in total enzyme per unit increase in cell mass (Monod, Pappenheimer & Cohen-Bazire, 1952), calculated as follows: let d_0 be initial culture density (mg. dry wt. of cells/ml.); d_t , density after time t ; Q_0 , initial enzyme activity ($Q_{\text{CO}_2}^{\text{N}}$); Q_t , enzyme activity after time t . Then: Differential rate = $(Q_t d_t - Q_0 d_0)/(d_t - d_0)$. This distinguishes between the effect of a compound on growth and its specific effect on enzyme formation. The results were corrected for enzyme formation in the absence of an exogenous inducer. Since the performance of different batches of culture varied slightly, the differential rate obtained with 10 mM-L-leucine was usually measured and taken as the standard.

Reagents. Amino acids were obtained from commercial sources. With the exception of L-leucine, L-lysine and L-isoleucine, DL-compounds were used in preference to L-isomers, since the DL-compounds are cheaper and are less likely to be contaminated with other amino acids. All were chromatographically pure except a cheap grade of L-leucine used only in preliminary experiments. DL-[1-¹⁴C]Valine and DL-[¹⁴C]leucine were purchased from The Radiochemical Centre, Amersham, Bucks. *N*-Acetyl-L-leucine was synthesized by the method of Syngé (1939). Pyridoxal phosphate was a generous gift from Roche Products Ltd., through the courtesy of Dr A. L. Morrison.

RESULTS

Conditions for induced enzyme formation

When *Proteus* was grown on the synthetic medium and dense, washed suspensions were incubated with valine in the presence of a source of carbon and nitrogen, there was an increase in the decarboxylase activity (Table 1). Omission either of valine or of the carbon source (lactate) abolished this increase in activity, but omission of ammonium chloride had no effect.

The decarboxylase activity induced in these washed suspensions was disappointingly low compared with the $Q_{\text{CO}_2}^{\text{N}}$ of 8.5 exhibited by cells which had been exposed to the inducer during growth on the synthetic medium with the addition of 0.5% of DL-valine. If, however, a culture of *Proteus* was allowed to grow on a simple lactate medium and an inducer added when growth was established but not complete, a few hours' further incubation yielded cells with higher decarboxylase values than those obtained in the experiments described in Table 1. If the data, given in Table 2, are plotted it is seen that the differential rate of enzyme formation remained constant for about 3 hr. but had fallen after 6 hr. to about half its initial value.

Glucose is known to inhibit the synthesis of many adaptive enzymes (Gale, 1945). When glucose (1%) was added to *Proteus* cultures together with the inducer (10 mM-leucine), decarboxylase formation was reduced by about 50%.

Table 1. *Induction of leucine decarboxylase in washed suspensions of Proteus vulgaris*

The complete system contained cells (4 mg./ml.), DL-valine (100 mM), sodium lactate (1%), NH_4Cl (0.1%), pyridoxal phosphate (0.5 $\mu\text{g.}/\text{ml.}$), nicotinamide (5 $\mu\text{g.}/\text{ml.}$) and phosphate buffer, pH 7.0 (0.04M). Flasks containing 10 ml. were shaken at 37° for 3 hr. The cells were then harvested and their decarboxylase activity was determined.

Conditions	Decarboxylase activity ($Q_{\text{CO}_2}^{\text{N}}$)
Complete system	3.8
Valine omitted	1.2
Lactate omitted	0.6
NH_4Cl omitted	4.1
Suspension before incubation	1.0

Table 2. *Induction of leucine decarboxylase in growing cultures of Proteus vulgaris*

Roux bottles containing 100 ml. of a culture of *Proteus* on synthetic medium were allowed to grow to a density of 0.81 mg. dry wt. of cells/ml. Leucine (25 ml.; 2.5%) was added at this stage ($t = 0$) and incubation continued for the further period stated. 2:4-Dinitrophenol (1 mM) was added to stop induction, the cells were harvested and washed, and their decarboxylase activity was determined.

Induction period (hr.)	Culture density (mg./ml.)	Decarboxylase activity ($Q_{\text{CO}_2}^{\text{N}}$)
0	0.81	2.75
1	0.86	5.6
2	1.05	8.6
3	1.16	9.3
4	1.24	8.7
5	1.29	11.0
6	1.32	10.5

Specificity of the enzyme

Various compounds structurally related to leucine were tested for their ability to act as substrates or inhibitors of leucine decarboxylase in cell-free extracts. Apart from the five amino acids already known to be substrates of the enzyme, none of the compounds tested were decarboxylated. Table 3 shows that α -aminoisobutyrate, α -aminobutyrate, lysine, alanine and phenylalanine also failed to inhibit the enzyme significantly. *N*-Acetyl-leucine, isobutylamine and isovalerate, however, inhibited the decarboxylation of leucine: the two last-named were shown to be competitive inhibitors and might therefore combine with the active centre of the enzyme. The nature of the *N*-acetyl-leucine inhibition was not investigated.

Samson, Katz & Harris (1955) reported that fatty acids of the chain length of isovalerate inhibit a number of enzymes, so it was necessary to consider whether the powerful inhibition of decarboxylation was due to a non-specific adsorption to the

surface of the enzyme protein. The kinetics of *iso*-valerate inhibition were therefore investigated by the method of Lineweaver & Burk (1934). Fig. 1 shows that inhibition was competitive, the dissociation constant (K_i) of the enzyme-*iso*valerate complex being 1.8 mm. It is evident that *iso*-valerate can compete with leucine for the substrate-binding site of the enzyme. Similar experiments were carried out with *n*-valerate and *n*-butyrate, valine being used as substrate in the latter case. Inhibition was competitive with these acids also. The K_i values were 2.9 mm for *n*-valerate and 17 mm for *n*-butyrate.

Table 3. *Inhibition of leucine decarboxylase by substrate analogues*

L-Leucine (58 mm) and the compound under test (58 mm-L-isomer) were added simultaneously or singly to cell-free extracts of broth-grown cells. All determinations were carried out in duplicate. The mean rate of CO_2 output in the absence of inhibitor was 37 $\mu\text{l./hr./mg.}$ of freeze-dried preparation, measured over a 40 min. period from addition of substrate.

Compound	Inhibition of leucine decarboxylation (%)
DL- α -Aminoisobutyrate	0
DL- α -Aminobutyrate	3
L-Lysine	8
DL-Alanine	6
DL-Phenylalanine	3
<i>iso</i> Butylamine	35
<i>N</i> -Acetyl-L-leucine	23
<i>iso</i> Valerate	85

Specificity of the induction system

The compounds previously examined for their ability to combine with leucine decarboxylase were also tested as inducers of the enzyme (Table 4). The differential rate of enzyme formation induced

Fig. 1. Lineweaver-Burk plot showing competitive inhibition of the decarboxylation of leucine over 40 min. period in cell-free extracts. The fatty acid (16.6 mm) and L-leucine were added simultaneously to the cell-free enzyme. O, No inhibitor; x, *n*-valerate; Δ , *iso*valerate.

Table 4. *Specificity of induction of leucine decarboxylase*

Compounds were tested at a concentration of 10 mm (20 mm for DL compounds) singly and in combination with L-leucine at two concentrations (10 and 1 mm). Results are expressed as the differential rate of enzyme formation (see Materials and Methods section), taking the rate induced by 10 mm-leucine as 100. The culture density increased by about 50% during the 3 hr. incubation period.

Compound	Rate of decarboxylase formation		
	With compound only	With compound + 10 mm-leucine	With compound + 1 mm-leucine
None	—	100	34
DL- α -Aminoisobutyrate	5	102	32
DL- α -Aminobutyrate	4	103	40
L-Lysine	6	93	26
DL-Alanine	32	90	68
DL-Phenylalanine	0	60	14
<i>iso</i> Butylamine	4	—	22*
<i>N</i> -Acetyl-L-leucine	63	110	83
<i>iso</i> Valerate	2	140	71
DL-Valine	72	117	78
DL- α -Amino- <i>n</i> -butyrate	70	115	83
L-Isoleucine	41	—	74

* In this experiment, 1 mm-leucine alone gave a value of only 22.

by leucine itself (10 mM) was 19.2, an average from 20 experiments. The s.d. of a single determination was 2.9, i.e. $\pm 15\%$. This makes it probable that the s.e.m. for the values given in Table 4 is about $\pm 10\%$, since each is the mean of at least two determinations.

Each substance was tested: (a) alone, to determine its own inducer action, if any, (b) in the presence of sufficient leucine (10 mM) to saturate the induction system, and (c) at a lower leucine concentration (1 mM), known to be well below the saturation level. Thus (b) would reveal whether an inducer was working through the same mechanism as leucine (an additive effect would be observed otherwise), whereas (c) offered favourable conditions for demonstration of competitive inhibition of induction.

α -Aminoisobutyrate, α -aminobutyrate and lysine were inactive as inducers, and did not affect induction by leucine. Alanine, however, which had very little affinity for the enzyme, showed considerable activity as an inducer. Presumably it acts through the same mechanism as leucine since its effect is additive with that of leucine only when the latter is present at a sub-saturation level. Of the compounds previously found to inhibit the enzyme, isobutylamine had no effect on induction, acetyl-leucine acted as an inducer, and isovalerate, although not itself an inducer, enhanced induction by leucine. Phenylalanine, which did not appear to combine with the enzyme, was also inactive as an inducer. However, when added simultaneously with leucine to growing cells, phenylalanine inhibited induction of the enzyme by leucine.

All the known substrates of the enzyme have been shown to induce its formation (Ekladius *et al.* 1957). Some are included in Table 4. The differential rate of enzyme formation obtained with α -amino-*n*-butyrate was of doubtful significance, as this compound almost completely inhibited growth, but the average $Q_{CO_2}^{N_2}$ induced was 2.4. Growth was inhibited slightly by DL-alanine and DL-phenylalanine also but not by any other compound tested.

Penetration of the cells

isoValerate was a potent inhibitor of the cell-free enzyme but failed to induce enzyme formation in growing cells. Before this was interpreted as a difference in specificity between the enzyme and the induction system, it was necessary to consider the alternative explanation that isovalerate might be unable to penetrate the cell. This was investigated by examining the effect of isovalerate on the decarboxylation of valine by intact cells (Table 5). At the concentration used in induction experiments, isovalerate caused marked inhibition of decarboxylation. This strongly suggests that it can penetrate the cells and gain access to the enzyme and therefore, presumably, to the induction system.

The inhibitory effect of phenylalanine on induction could mean that it combines with the induction system, forming an inactive complex. Alternatively, it might prevent access of leucine to the induction system, e.g. by inhibiting penetration of leucine into the cell. This possibility was examined by adding DL-phenylalanine (20 mM) and L-leucine (20 mM) simultaneously to washed suspensions of broth-grown *Proteus* under the usual conditions of decarboxylase assay. The leucine concentration was insufficient to saturate the decarboxylase system, so a low intracellular leucine concentration should have been manifested as a low rate of decarboxylation if phenylalanine inhibited entry of leucine into the cells. No significant inhibition of decarboxylation was observed.

Table 5. *Effect of isovalerate on decarboxylation of valine by intact cells*

A washed suspension of broth-grown *Proteus* was used as enzyme preparation. Each flask contained 18.6 mg. of cells. Production of CO_2 was measured over a period of 40 min. from addition of substrate.

Substrate	$Q_{CO_2}^{N_2}$
None	1
DL-Valine (100 mM)	21.8
isoValerate (10 mM)	1
Valine (100 mM) + isovalerate (10 mM) (added simultaneously)	14.3

Table 6. *Effect of phenylalanine on induction of leucine decarboxylase by alanine*

DL-Alanine (20 mM), with or without DL-phenylalanine (20 mM), was added to *Proteus* growing on lactate medium. The decarboxylase activity of the cells was determined after incubation for 3 hr.

	Initial	Final	
		Alanine added	Alanine + phenylalanine added
Culture density (mg./ml.)	0.63	0.86	0.73
Enzyme activity ($Q_{CO_2}^{N_2}$)	1.8	5.7	3.9
Differential rate of decarboxylase formation	—	16.4	17.2

This experiment was, however, carried out with broth-grown cells in buffer, pH 6, under anaerobic conditions. Cells growing in a synthetic complex medium under aerobic conditions might behave differently. The effect of phenylalanine on induction by alanine was therefore investigated. (Alanine was chosen because it is the inducer that least resembles leucine.) Although the decarboxylase activity induced was lower in the presence of phenylalanine, this was due to a non-specific retardation of growth (Table 6). Phenylalanine had no specific effect on the differential rate of decarboxylase formation induced by alanine. This suggests that phenylalanine does not combine with the induction system, but interferes with leucine at some other site.

Affinity of some amino acids for the enzyme and for the induction system

Substrate affinity. The affinity of the decarboxylase for four of its substrates was investigated with cell-free extracts of broth-grown cells. The Lineweaver-Burk plots were linear and the Michaelis constants (K_m) were about 10 mM for each amino acid tested (Table 7).

When an enzyme is present in an intact cell, its apparent affinity for its substrate may differ from that of the cell-free enzyme. This may be due to changes in the enzyme itself during extraction from the cell. Another important factor is that the cell boundaries may either impede or facilitate the entry of substrates into the cell, so leading to differences between the intracellular and extracellular concentrations. The effect of substrate concentration on the rate of decarboxylation by intact cells of *Proteus* was therefore investigated. Lineweaver-Burk plots gave apparent Michaelis constants with leucine, isoleucine and norvaline similar to those found with cell-free extracts, suggesting that the intracellular concentration was in each case similar to the extracellular concentration. With valine, however, the intact cells gave the apparent Michaelis constant as about 30 mM, suggesting that valine did not enter the cells freely and that the intracellular concentration was less than the extracellular concentration for the range tested.

Penetration of cells by substrate inducers. Induction takes place within the cell, but the concentration of inducer can be determined readily only in the external medium. The inducer constants given in column 3 of Table 7 are a basis for valid conclusions only if the intracellular concentrations of the inducers in the appropriate experiments approximated to those in the external medium. Cohen & Rickenberg (1956) have suggested that some amino acids may be concentrated within the

Table 7. *Comparison of apparent dissociation constants for the action and induction of leucine decarboxylase*

The apparent Michaelis constants for leucine decarboxylase were deduced from the data of Figs. 2 and 3. The last column gives the apparent dissociation constant of the complex formed between an inducer and the site in the cell which combines with it, assuming that Michaelis-Menten kinetics can be applied (see Figs. 2 and 3).

Amino acid	Apparent K_m		Inducer constants (mM)
	Cell-free extracts (mM)	Intact cells (mM)	
Leucine	9	11	1.1
Valine	11	30	0.8
Isoleucine	11	8	1.4
Norvaline	9	9	—

cell by specific enzymes or 'permeases'. Whilst neither subscribing to this hypothesis nor rejecting it, the present authors recognize the necessity for considering it in relation to the system under investigation.

Although the comparison of apparent Michaelis constants in intact cells and cell-free extracts, described above, does not suggest any concentration of substrates within the cell, permease action may require the presence of an energy source. Manometric determination of decarboxylase activity may not be feasible if other substances are being metabolized, so internal concentrations of valine and leucine were investigated directly by means of isotope-labelled material.

The organism was grown on nutrient broth (to provide opportunity for formation of inducible amino acid permeases); washed suspensions were prepared and exposed, in the presence of glucose, to generally labelled [^{14}C]valine or [^{14}C]leucine. After 10 min. at 37° or 30 min. at 0° the cells were centrifuged down at 0°, washed with ice-cold water, then suspended in a small volume of water and heated to 100° for 3 min. After heating, the suspension was centrifuged and the radioactivity of the supernatants determined (only slight activity was found in the extracted cells). The radioactivity of the incubation medium was also determined.

The results are given in Table 8: here it is assumed that a cell space of 1 ml. will contain 0.2 g. dry wt. The intracellular concentrations of leucine and valine appear to be below those of the external medium. There is little difference between the results obtained at 37° and at 0°, where no enzymically assisted entry would occur. The external substrate concentrations chosen, 10 mM and 1 mM, covered the range over which most of the induction experiments were performed.

Table 8. Uptake of [^{14}C]leucine and [^{14}C]valine by washed cells of *Proteus vulgaris* in the presence of glucose

Tubes containing washed cells (30–40 mg. dry wt.), glucose (0.4%), generally labelled [^{14}C]leucine or valine (10 mM or 1 mM; 0.5 μC) in a total volume of 5 ml. of KH_2PO_4 – Na_2HPO_4 buffer (0.05M; pH 7) were incubated at 37° for 10 min. or at 0° for 30 min. Cells were then centrifuged at 0° and extracted by heating at 100° (see text). The supernatant medium and cell extracts were assayed for radioactivity in an end-window counter: results are corrected to zero thickness and expressed as counts/min./ml. of supernatant medium or cell volume (taken as 5 $\times$ dry wt.).

External concn.	...	...	Leucine			
			10 mM		1 mM	
Incubation conditions	...	...	0°/30 min.	37°/10 min.	0°/30 min.	37°/10 min.
Medium			8080	7340	7480	6560
Cells			1300	1800	1920	2650

External concn.	...	...	Valine			
			10 mM		1 mM	
Incubation conditions	...	...	0°/30 min.	37°/10 min.	0°/30 min.	37°/10 min.
Medium			8280	7850	9140	8500
Cells			1890	3080	2850	2960

Fig. 2. Effect of L-leucine concentration on decarboxylase induction. The differential rate of decarboxylase formation (V) is expressed as a fraction of the induction by 10 mM-leucine (19.2 units). Each point represents the mean of several determinations: the results from a total of eight series of experiments are included, and the s.d. of each point is indicated.

These experiments provide no grounds for suggesting that the internal cell concentrations of valine and leucine significantly exceeded the external values under the conditions of the induction experiments.

Fig. 3. Effect of inducer concentrations on decarboxylase induction. The units are the same as in Fig. 2. $\circ$, L-isoleucine; $\bullet$, DL-valine (concentrations given refer to the L-isomer). The rate of formation of decarboxylase with 10 mM-leucine is taken as 1.

Affinity of the induction system for inducers. The effect of leucine concentration on induction was tested in a preliminary experiment; above a concentration of 20 mM, leucine inhibited induction slightly, so this was the highest concentration used in further experiments. Figs. 2 and 3 show the effect of the concentration of leucine, valine and isoleucine on induction. The reciprocal plots are linear, suggesting that the combination of inducers with the induction system obeys Michaelis-Menten kinetics. The induction constants, analogous to the

Table 9. *Effect of alanine concentration on induction*

Induction was carried out in the usual way, with DL-alanine as inducer. The decarboxylase activity of the washed cells was measured by the isotopic method, with about 18 mg. of cells per Conway unit. The differential rate of decarboxylase formation is expressed as a percentage of the rate (7.95) obtained with 20 mM-DL-alanine. During the induction period the culture density increased from 0.72 to 1.01 mg./ml., except at the highest alanine concentration when the density rose only to 0.97 mg./ml.

Concn. of L-alanine (mM)	Decarboxylase formation (%)
40	70
20	82
10	100
2	47

Table 10. *Disappearance of leucine from cultures*

For details see text.

Initial L-leucine concn. (mM)	Amino acid concn. after 3.5 hr. (mM)
20	8.6
10	3.8
5	1.5
2	0.5
1	0.4
0	0.3

Michaelis constant of an enzyme, have been given in Table 7.

The effect of alanine concentration on decarboxylase formation is shown in Table 9. DL-Alanine concentrations above 20 mM appeared to inhibit induction.

The inducers used were metabolized by *Proteus* under the conditions of induction. In the experiments described above, the concentrations given are the initial concentrations and the 'induction constants' are therefore maximum values. To gain some indication of the rate of disappearance of leucine, cultures were incubated with various concentrations of leucine under the conditions employed in the induction experiments. The initial cell density was 0.7 mg./ml. After 3.5 hr. the cells were removed by centrifuging and the α -amino nitrogen of the supernatants was estimated by the method of Van Slyke (1929). Table 10 shows that virtually all the leucine disappeared from the medium if the initial concentration was 2 mM or less.

DISCUSSION

Decarboxylase activity in cells after exposure to an inducer might arise in one of two ways: (a) a constitutive decarboxylase might be always present, but be called into action only when an in-

ducible permease is formed to concentrate the substrate (or possibly the coenzyme) to a level which would permit interaction with the apoenzyme; (b) induced formation of the decarboxylase itself. Ekladius *et al.* (1957) found no appreciable decarboxylase activity, even in the presence of excess of coenzyme, when *Proteus* was grown in the absence of an inducer and then mechanically disintegrated. When grown in the presence of any of five inducers, however, decarboxylase activity appeared that was substantially the same level for cell-free extracts as for whole cells, in respect of each of five substrates. These findings supported the concept of a true inducible decarboxylase. Moreover, if permeases had been playing a significant part in the overall decarboxylation process, significantly lower apparent Michaelis constants would be expected for intact cell as compared with cell-free enzyme preparations. This was not the case (Table 7). Similar reasoning, applied to the measurements of apoenzyme-coenzyme affinities in intact cell and cell-free preparations (Ekladius *et al.* 1957) would reject the suggestion that the results presented here can be explained on the basis of an inducible coenzyme permease.

We therefore considered decarboxylase levels in the intact cells under appropriate conditions an adequate measure of enzyme activity. Use of cell-free extracts at all stages might have been theoretically preferable, but would have considerably multiplied the technical difficulties, which were already formidable. In any case, the experiments with [^{14}C]valine and [^{14}C]leucine (Table 8) yielded no evidence of concentration of these amino acids within the cell under the conditions of our induction experiments.

Leucine decarboxylase has many common features with the receptor with which the inducers combine. Any of the five known substrates will induce enzyme formation and their efficiency runs roughly parallel with their activity as substrates. Ekladius *et al.* (1957) report the Q_{00}^N values of cell-free extracts of broth-grown cells as follows: leucine, 37; valine, 24; norvaline, 23; isoleucine, 18; α -aminobutyric acid, 13. Induction activity is rated both by ourselves (Table 4) and by Ekladius in the order leucine, valine, α -aminobutyrate, isoleucine, although Ekladius's results were obtained under different conditions and are not quantitatively comparable with ours.

The specificity of the induction system differed, however, from that of the enzyme system, as for β -galactosidase investigated by Monod *et al.* (1951). Both *isovalerate* and *isobutylamine* are competitive inhibitors of leucine decarboxylase and hence, presumably, can combine with the substrate-binding centre; yet neither can serve as inducer although *isovalerate* could certainly penetrate the

cell. On the other hand, there is good reason to believe that the amino acid substrates combine with the enzyme through both their carboxyl and amino groups (Sutton & King, 1959), and the complexes formed between the enzyme and *isobutylamine* or *isovalerate* may not be strictly comparable with those formed with the amino acids themselves. *isovalerate*, however, has the remarkable property of potentiating induction by leucine. We do not know the mechanism involved.

Alanine provides a more clear-cut example of the difference in specificity between the enzyme and induction systems. There is also no permeability problem here. It is fairly active as an inducer, though apparently unable to combine with the enzyme itself as it functions neither as a substrate nor as an inhibitor.

The behaviour of phenylalanine, however, is puzzling. It inhibits induction by leucine though it does not appear to combine with the enzyme. On the other hand, it has no effect on enzyme formation when the latter is induced by alanine. Phenylalanine does not interfere with the penetration of leucine into *Proteus* under our experimental conditions, so we could exclude competitive interference with any 'permease' system such as that which catalyses entry of leucine into *E. coli* (Cohen & Rickenberg, 1956). Even these authors, however, found no interference with leucine entry by phenylalanine. The effect of phenylalanine in inhibiting induction by leucine but not by alanine remains unexplained.

Determination of the apparent affinities of the inducer for the induction receptor is complicated by the metabolic removal of the inducers (Table 10). It is particularly difficult to ensure reasonably constant inducer concentrations throughout the 3 hr. induction period at low inducer concentrations when the activity being measured is, in any case, small. The effect, however, would seem to be less serious than appears at first sight since the reciprocal plots in Figs. 2 and 3 are linear throughout the range of inducer concentrations recorded. Serious inducer exhaustion at the lower dilutions would have yielded plots with a concave form and given affinity constants greater than the true values. The constants deduced from the plots therefore represent maximum values, and it seems that leucine, isoleucine and valine have affinities for the induction system not less than ten times as great as their respective affinities for the enzyme itself. Conditions of induction in growing cells are admittedly different from those obtaining in the cell-free extracts used for the study of enzyme action, but induction in cell-free systems has unfortunately not proved possible in this system.

Our results, however, accord with those reported for other systems. Landman (1957) found the

affinity of methyl β -galactoside much lower for β -galactosidase than for the induction system for that enzyme; Pollock (1957) found that the affinity of various substrates and inhibitors for penicillinase was 10–100 times smaller than their affinity for the corresponding induction system. These results all support the view that the receptor with which an inducer interacts is not the enzyme itself, as was suggested by Yudkin (1938).

Another important feature of induction is that neither the specificity of the enzyme, nor the relative rate at which it attacks its various substrates, is influenced by the nature of the inducer employed (Ekladius *et al.* 1957). Commenting on this phenomenon in other systems, Cohn & Monod (1953) proposed that all the inducers of a given enzyme are metabolized to a common intermediate that is the true promoter of enzyme formation. It is manifestly unlikely, however, that the amino acid inducers of leucine decarboxylase would be metabolized to a common primary inducer, although it is probably true that all combine with the same receptor site on the induction system. On the other hand, the enzyme-forming activity does depend on the inducer employed. Figs. 2 and 3 show that the theoretical maximum rate of decarboxylase formation attainable even at saturating concentrations of inducer is greater with leucine than with isoleucine. Similarly, Pollock (1957) found greater maximal induction of penicillinase by cephalosporin C than was obtained with other inducers of this enzyme.

The most remarkable feature of our own investigations, and of similar studies by other workers with different systems, is the paradox of striking similarity, yet lack of identity, between the enzyme itself and the receptor involved in its induction. We were forced to conclusions similar to those reached by Monod (1958). The essential feature of this concept is that inducers combined with a bound form of the enzyme. It would be in accordance with current biochemical thought to suggest that this might be an alignment of the constituent amino acids on the surface of a nucleic acid template. The inducer might then interact with this complex in such a manner as to secure its release from the template and perhaps influence its folding in such a way as to determine, in part, its specificity (Halvorson, 1960). This mechanism would account for the close approximation between the specificities of induction and enzyme action, without requiring precise identity. If the combination of the free enzyme with its substrate caused any increase in the rate of enzyme synthesis by a direct 'mass-action' effect this would be a secondary action of the inducer and one which, in the case of leucine decarboxylase, does not appear to have any significant effect on the overall process.

SUMMARY

1. *Proteus vulgaris* possesses an inducible leucine decarboxylase. The formation of this enzyme was dependent on a source of carbon and energy in addition to an inducer, and was more rapid and extensive in growing cultures than in dense, washed suspensions.

2. A variety of compounds structurally related to leucine were tested for their ability to combine with the enzyme (acting as substrates or inhibitors) and to induce its formation. All the known substrates were inducers. *iso*Valerate and *isobutyl*-amine, which inhibited the enzyme, could not induce its formation, whereas alanine was an inducer but was inert in the enzyme system.

3. Those amino acids which were both substrates and inducers of the enzyme showed an affinity for the induction system at least ten times as great as their affinity for the enzyme.

4. The induction system closely resembles the enzyme system but is distinct from it. This is in keeping with the theory that inducers combine with a bound form of the enzyme.

We are grateful to Professor R. A. Morton, F.R.S., for his interest in this work. The Medical Research Council provided a grant towards expenses and scholarship for one of us (B.G.H.).

REFERENCES

- Cohen, G. N. & Rickenberg, H. V. (1956). *Ann. Inst. Pasteur*, **91**, 693.
 Cohn, M. & Monod, J. (1953). *Symp. Soc. gen. Microbiol.* **3**, 132.
 Ekladius, L., King, H. K. & Sutton, C. R. (1957). *J. gen. Microbiol.* **17**, 602.
 Gale, E. F. (1940). *Biochem. J.* **34**, 392.
 Gale, E. F. (1945). *Bact. Rev.* **7**, 139.
 Gale, E. F. (1957). *Meth. biochem. Anal.* **4**, 285.
 Halvorson, H. O. (1960). *Advanc. Enzymol.* **22**, 99.
 Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
 King, H. K. & Alexander, H. (1948). *J. gen. Microbiol.* **2**, 315.
 Landman, O. E. (1957). *Biochim. biophys. Acta*, **23**, 558.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Monod, J. (1958). *Rec. Trav. chim. Pays-Bas*, **77**, 569.
 Monod, J., Cohen-Bazire, G. & Cohn, M. (1951). *Biochim. biophys. Acta*, **7**, 585.
 Monod, J., Pappenheimer, A. M. & Cohen-Bazire, G. (1952). *Biochim. biophys. Acta*, **9**, 648.
 Pollock, M. R. (1957). *Biochem. J.* **66**, 419.
 Samson, F. E., Katz, A. M. & Harris, D. L. (1955). *Arch. Biochem. Biophys.* **54**, 406.
 Sutton, C. R. & King, H. K. (1959). *Biochem. J.* **73**, 43 p.
 Synge, R. L. M. (1939). *Biochem. J.* **33**, 1913.
 Van Slyke, D. D. (1929). *J. biol. Chem.* **83**, 425.
 Yudkin, J. (1938). *Biol. Rev.* **13**, 93.

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The Rate of Incorporation of [³⁵S]Methionine and [³⁵S]Cystine into Proteolipids and Proteins of Rat Brain

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(Received 5 January 1961)

Gaitonde & Richter (1956) have reported that, at 3 hr. after intracisternal injection of [³⁵S]methionine, about 17 % of the radioactivity in the brain was found in the trichloroacetic acid-soluble fraction, 3 % in the lipids and 80 % in the proteins. The specific radioactivities of the three fractions gave evidence of a rapid turnover of labelled methionine in the proteins. The specific radioactivity of the lipid sulphur was low and no definite conclusions were drawn as to the metabolic activity of this fraction; but it was observed that the labelling of lipid sulphur was mainly due to small amounts of lipoproteins present in the lipid extracts.

By extracting fresh ox-brain tissue with chloro-

form-methanol (2:1, v/v), Folch & Lees (1951) obtained a group of lipoproteins which they designated as proteolipids because of their solubility in lipid solvents. The proteolipid content of the white matter was three to four times that of the grey matter of the brain. Measurable quantities of proteolipids are present in the 7- or 8-day-old mouse brain. The proteolipid content of the brain increases greatly during the period of myelination, and in the 30-day-old mouse it reaches the level in the adult brain (Folch, 1954). These results suggest a possible significance of proteolipids in relation to the formation of myelin. To obtain further information on this point a study has now been made of the turnover of the proteolipids of rat brain at