ADVANCES IN ENZYMOLOGY

AND RELATED SUBJECTS OF BIOCHEMISTRY

Edited by F. F. NORD

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AFTER FIVE YEARS

When *Ergebnisse der Enzymforschung* became extinct, *Advances in Enzymology* was initiated as an outlet for the publication of critical reports abutting on the borderland between physiology, chemistry, microbiology and physical chemistry. The continued participation of this organ in keeping abreast of our vast subject has again resulted in a volume which, as has been the endeavor since its inception, approaches the true *polis* of the enzymologist.

It is also intended in the future to rotate and expand the subjects reported, at the same time limiting the articles to topics that are reasonably ready for critical discussion.

Volume VI is presented to the reader about one hundred years after separation from the living cell of the diastases by Payen and Persoz and of pepsin by Schwann. It is my sincere hope that the cessation of the armed hostilities will help to restore the community spirit and consolidate our efforts in our field in which the *Advances* have not as yet completed their mission.

THE EDITOR

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THE BACTERIAL AMINO ACID DECARBOXYLASES

By

ERNEST F. GALE

Cambridge, England

CONTENTS

I. Production of Amines by Bacteria

The production of amines as a result of bacterial putrefaction has been recognized since the beginning of the present century (9, 53), and Ellinger (19, 20) and Ackermann (1–4) showed that if media consisting of inorganic

salts, peptone, glucose, and certain amino acids are inoculated with putrefying material and the medium examined chemically after a period of some weeks, the amines corresponding to these amino acids can be isolated in fair yield. In this way the biological production of putrescine, histamine, cadaverine, tyramine, β-alanine, γ-aminobutyric acid, and δ-aminovaleric acid from the corresponding amino acids was demonstrated. Pure strains of organisms were used for the production of histamine from histidine by Mellanby and Twort (51) and Berthelot and Bertrand (12). The organism isolated by the latter workers proved capable also of forming tyramine and tryptamine from tyrosine and tryptophan, respectively, and was accordingly named *Bacillus aminophilus intestinalis*. Other workers followed the formation of amines in culture by the growth of pure strains of various bacteria and found that *Escherichia coli* can produce histamine or tyramine (41, 42, 54), putrescine from arginine (5), and isoamylamine from leucine (6); *Bacillus mesentericus vulgatus* can form putrescine, cadaverine, and tryptamine (37), and many genera and species can form histamine (17, 41, 44, 46, 47, 51).

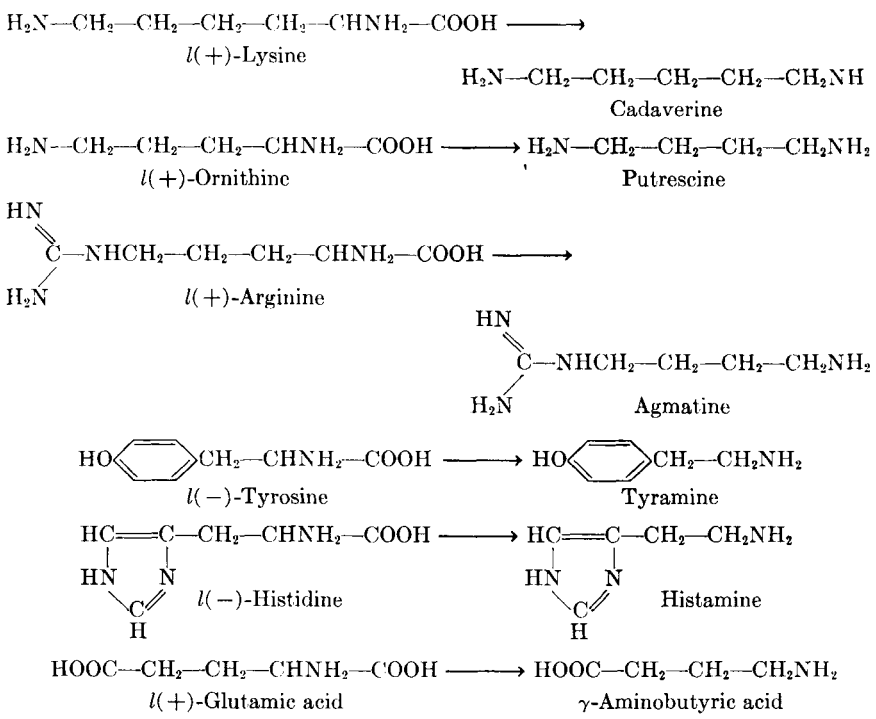
The greater part of this early work serves to emphasize the biological nature of amine production but gives little information concerning the mechanism of or the conditions necessary for such metabolism. It is noticeable that, in the majority of these cases, amines are produced only when fermentable carbohydrate is present in the growth medium. Koessler and Hanke (48) carried out a detailed investigation of the formation of histamine by a "colon bacillus" using an extraction method to remove histamine from culture prior to colorimetric estimation. They showed that whenever the amine is produced, the medium first becomes distinctly acid and that "histamine is never formed except in the presence of an easily available source of carbon such as glycerol or glucose." The investigations were extended to other "colon bacilli" (41, 42) and the same general conclusion was reached. It was found that some of these organisms were able to form tyramine when grown in a medium containing tyrosine and fermentable carbohydrate, but that the organisms which produce tyramine would not produce histamine and vice versa. These authors made the further interesting discovery that those organisms which attacked tyrosine to form tyramine when the growth medium was allowed to become acid, also attacked the amino acid in an alkaline medium, but the product was now phenol and not tyramine. Eggerth and co-workers (18) improved the methods of extraction and estimation of histamine in cultures and studied (17) the formation of histamine by many strains of various genera including *E. coli*, *Salmonella*, *Eberthella*, *Aerobacter*, *Clostridium welchii*, etc. The organisms were grown in various determined media containing inorganic salts, glucose, and, in some cases, asparagine or peptone to assist growth. In positive cases, histamine formation from histidine began within 24 hours and continued for 4 to 5 days. Experiments were carried out in which the *p*H of the media was controlled and it was found that histamine production was most rapid when the medium *p*H lay between 5.0 and 5.5 and was markedly, in some cases completely, inhibited by medium *p*H values higher than 6.5. With some strains it was found that temperature affects the histamine formation, organisms such as certain

strains of *E. coli* and *Aerobacter aerogenes* having optimal temperatures below 30° C. while others, such as *Cl. welchii*, displaying optimal histamine production at growth temperatures around 37°.

Gale (24–27) showed that amines are produced by bacteria through the action of specific amino acid decarboxylases and that these enzymes are formed within bacterial cells in response to certain well-defined conditions of growth. The enzymes have been obtained in a cell-free condition (21, 22, 32, 65) and their properties have now been investigated in detail. The present article summarizes the knowledge that has been gained of the nature, action, formation, and distribution of these bacterial amino acid decarboxylases.

II. Decarboxylation of Amino Acids by Bacteria

The simple decarboxylation of an amino acid results in the formation of the corresponding amine with the liberation of carbon dioxide. If the reaction takes place at an acid *p*H value, the carbon dioxide is evolved in the gas phase and the course of the reaction can be followed manometrically. The bacterial amino acid decarboxylases, so far identified, all react at optimum *p*H values lying 2.5 to 6.0, so that when washed suspensions of bacteria possessing such enzymes are shaken with the appropriate amino acid substrate in a manometer, amine production is indicated by an evolution of carbon dioxide. During recent years many bacterial genera and species have been tested under suitable experimental conditions for their power to decarboxylate the common amino acids; so far only six amino acids have been attacked in such experiments (see Scheme 1). These six amino acids have one property in common: that, in addition to the terminal —COOH group and the α-NH_2 group, they all have a further polar group situated at the end of the molecule removed from the —COOH group attacked. That the integrity of this third polar group is necessary for decarboxylation to occur is indicated by the fact that if the group is substituted or altered, then the enzyme can no longer attack the molecule concerned. Thus *l*(+)-lysine decarboxylase cannot attack lysine in which either NH_2 group has been methylated or benzoylated; *l*(−)-tyrosine decarboxylase cannot attack tyrosine in which the —OH group has been methylated or benzoylated and is inactive toward phenylalanine; *l*(+)-glutamic acid decarboxylase cannot attack any peptide containing glutamic acid whether the peptide linkage attaches to the 1 or 5 position. In each case the enzyme concerned is specific for the natural isomer of the amino acid substrate. Substitution of the α-NH_2 group results in complete



SCHEME 1

inhibition of the attack and the corresponding keto acids are not attacked. Since $l(+)$-lysine decarboxylase cannot decarboxylate $l(+)$-ornithine, the length of the carbon chain between the polar groups is also of importance.

These findings, considered in conjunction with the fact that no monoaminomonocarboxylic acid is attacked in this way, suggest that for an amino acid to evoke the formation of a decarboxylase in bacteria it must possess: (*1*) a free COOH group in the 1 position; (*2*) a free α-NH_2 group; (*3*) a free terminal group of polar nature; and (*4*) the natural *levo* configuration.

It would seem that, for an enzyme to produce the strain necessary to result in the splitting off of carbon dioxide from the —COOH group of the substrate molecule, there must be a two-point attachment between the substrate and the enzyme protein, and the presence of polar groups in the right stereochemical relation is necessary for the evocation of the enzymes during growth.

The question arises whether the reverse condition holds true: can any

amino acid with two polar groups other than the —COOH group evoke the formation of a decarboxylase in some organism or other? If this should be the case, then we should expect to find bacteria which will attack aspartic acid or tryptophan. Virtanen and Laine (69) have claimed that *Rhizobium leguminosarum* decarboxylates aspartic acid with the formation of β-alanine, while Ackermann (1–4), in the course of his studies on the production of amines during putrefaction, isolated β-alanine from media containing aspartic acid, so that it is probable that bacteria do occur which will carry out this decarboxylation. There are also claims in the literature (12, 37) that tryptamine has been obtained by the action of bacteria on tryptophan, but studies in this unit involving a survey of some 200 coliform organisms, 800 streptococci, 30 clostridia, and representative strains of *Pseudomonas*, *Proteus*, *Bacilli* (including *B. mesentericus*, *B. subtilis*, etc.), etc., have so far not yielded any organism which can decarboxylate any common amino acid other than the six listed above. All these studies have involved the use of washed suspensions and a manometric technique of following decarboxylation and it may be that very slow decarboxylations, which would yield a product over a long period of incubation in a growth experiment, would not appear significant in the washed suspension investigations.

The activities of amino acid decarboxylases quoted in this paper are expressed as Q_{CO_2} = μl. CO_2 liberated from substrate at 30° C. and at the optimum *p*H per hr. per mg. dry weight of organism.

III. Conditions Necessary for Formation of Amino Acid Decarboxylases in Bacteria

For the formation of active amino acid decarboxylases in bacteria the following conditions have to be fulfilled:

1. The organism concerned must possess such enzymes in its potential enzymic constitution.
2. Growth must take place in the presence of the specific substrate.
3. The organism must be capable of synthesizing codecarboxylase or, if the organism cannot accomplish this synthesis, the growth medium must contain certain factors involved in the formation of codecarboxylase.
4. The growth medium must be acid.
5. With some organisms, amino acid decarboxylases are formed to a significant extent only if growth occurs at temperatures lower than 30° C.
6. The enzymes are fully developed within the organism only at the end of active cell division.

These conditions will now be considered in detail.

1. *Distribution of Amino Acid Decarboxylases in Potential Enzymic Constitution of Bacteria*

The potential enzymic constitution of an organism is the sum total of all the enzymes that that particular organism can produce when grown under suitable conditions and represents the enzymic repertoire of the organism from which the actual enzymic constitution of any particular cell or culture is selected by the conditions of growth and formation of that cell or culture (28). All bacteria cannot produce all six known decarboxylases; in fact it is probable that the majority of bacteria are unable to produce these enzymes at all. In recent years the author and his colleagues have investigated the distribution of the six amino acid decarboxylases among the potential enzymic constitutions of various bacterial genera and species. The main results can be summarized as follows.

***Escherichia coli* and Related Organisms.**—Most coliform organisms when grown under suitable conditions can form amino acid decarboxylases. Of 151 strains of *E. coli* investigated, 114 have possessed arginine decarboxylase, 142 lysine decarboxylase, 130 glutamic acid decarboxylase, 14 histidine decarboxylase (a larger number may have possessed this enzyme in a weakly active state but generally organisms whose activity (Q_{CO_2}) has been less than 2 to 3 have been recorded as negative), and 6 strains have possessed tyrosine decarboxylase. Many of these organisms have not been tested against ornithine, but of those tested, 90% have possessed ornithine decarboxylase. No organism has been found which possesses all six decarboxylases but any number from 5 to 0 may occur in specific strains. Tyrosine decarboxylase occurs rarely among coliform organisms. Stadler and Neus (63) found that 9 out of 100 strains of *E. coli* possessed this enzyme and it has also been recorded in this group by Hanke and Koessler (42). The finding of these last workers that the tyrosine and histidine enzymes are not found together appears to be confirmed. No one enzyme ever occurs in constant association with any other. The activities of the enzymes when the cells are grown and tested under optimal conditions varies anywhere between $Q_{CO_2} = 1$ and 1200, being of the order 200 to 300 for the arginine, lysine, and glutamic acid enzymes in most cases, and less than 100 for histidine decarboxylase. Other organisms having similar distributions of these enzymes are *Aerobacter aerogenes* and *Klebsiella pneumoniae.*

Streptococci.—Some 800 strains of streptococci have been examined for decarboxylase activity. Approximately 500 strains of *S. faecalis* or streptococci belonging to Lancefield's group D have possessed tyrosine decarboxylase to a variable degree, but no other amino acid decarboxylase.

Representative strains of streptococci belonging to Lancefield's groups A to F have all shown tyrosine decarboxylase activity, but no other. A limited number of *S. lactis* strains have been tested and have shown no decarboxylase activities. The activities of the tyrosine enzymes vary widely with strain, the majority of *S. faecalis* strains from normal healthy infants having Q_{CO_2} values less than 50, while organisms isolated from certain diarrheal conditions have Q_{CO_2} values 200 to 300.

Clostridia.—Of ten strains of *Cl. welchii* type A examined, nine possessed histidine decarboxylase and glutamic acid decarboxylase, while the classical strain S.R.12 possessed only glutamic acid decarboxylase. Strains of *Cl. welchii* types B, C, and D possessed both decarboxylases. Four strains of *Cl. septicum* possessed ornithine decarboxylase only and this enzyme was present in a highly active state in the four organisms. Two strains of *Cl. bifermentans* possessed glutamic acid decarboxylase, one of two strains of *Cl. fallax* possessed histidine decarboxylase, and *Cl. aerofoetidum* had both tyrosine and glutamic acid enzymes. Representative strains of many other species of *Clostridia* (*sporogenes, histolyticum, chauvoei, oedematiens, tetani, butyricum,* etc.) were inactive.

Proteus.—Some twenty strains of *Proteus vulgaris, morganii,* etc., have been tested. The majority possess both glutamic acid decarboxylase and ornithine decarboxylase but some strains may possess either or none.

Bacilli.—No detailed investigation has as yet been undertaken but preliminary investigations show that *B. subtilis* and *B. mesentericus* strains possess weak arginine, lysine, ornithine, and glutamic acid enzymes.

General.—No active enzmyes have been found in organisms belonging to *Staphylococcus* or *Pseudomonas. Lactobacilli* have not yet been examined. In all cases tests have been made on the whole range of common amino acids (24) but in no case, so far, has any organism been discovered which could decarboxylate any amino acid other than the six listed above, under the experimental conditions used.

2. Adaptation to Substrate

Table I shows the activity toward the six amino acids of various strains of *E. coli,* grown in a simple salt–glucose–ammonia medium. When these organisms are grown in such a medium free of amino acids, the amino acid decarboxylases, with the exception of the glutamic acid enzyme, are not produced. The addition of the amino acid substrate to the growth medium results in the production of the corresponding decarboxylase (provided that this is present in the potential enzymic constitution of the organism tested). Thus the presence of 1% lysine in the growth medium results

in the production of an organism having a Q_{CO_2} (lysine) of 210 compared with the value of 194 for the same organism growing in the fully nutrient casein digest–glucose medium. In the same way the addition of 1% ornithine to the growth medium results in the adaptive formation of ornithine decarboxylase. Similar results are obtained with the tyrosine and histidine enzymes. Arginine decarboxylase formation apparently requires factors other than simple substrate adaptation as the addition of 1% arginine to the growth medium results in an organism having a Q_{CO_2} (arginine) of 27 compared with the value of 330 when growth takes place in the digest medium. The glutamic acid decarboxylase is formed to some extent in the simple amino acid-free medium, but the activity is doubled by the addition of specific substrate during growth. There is adaptive formation of all these enzymes in response to the presence of the specific substrate during growth but the full development of the arginine decarboxylase apparently requires other factors.

TABLE I

ADAPTIVE FORMATION OF AMINO ACID DECARBOXYLASES* IN *Escherichia coli*†

Decarboxylase	*E. coli* strain	Additions to medium							
		None	Lysine	Arginine	Ornithine	Glutamate	Histidine	Tyrosine	Casein digest
l(+)-Lysine	86	4	210	..	4	..	..	..	194
l(+)-Arginine	86	0	...	27	...	..	..	..	330
l(+)-Ornithine	86	3	...	..	225	..	..	..	145
l(+)-Glutamic acid	TY	45	...	..	...	88	..	..	100
l(−)-Histidine	86	0	...	..	...	..	7	..	18
l(−)-Tyrosine	HE	0	...	..	...	..	..	60	63

* Growth medium: inorganic salt mixture, including $(NH_4)_2HPO_4$, + 2% glucose + additions (1%) as above.

† Activities expressed in values of Q_{CO_2} at 30° C. and optimum *p*H.

3. *Codecarboxylase Factors in Growth Medium*

Gale (25) found that strains of *Streptococcus faecalis* possess a very active tyrosine decarboxylase but that, when these organisms are grown in a simplified medium, the activity of the resulting organisms is greatly decreased. For example, a strain of *S. faecalis* grown in casein digest–glucose has a Q_{CO_2} (tyrosine) of 218, but when grown in a salt–glucose–marmite medium the activity is 8 and is raised to 20 by the addition of 1% tyrosine to the growth medium. There was adaptation to substrate in this case, but the resulting activity was considerably less than that developed in the complex casein digest medium. It was suggested (28)

that the organism is unable to synthesize some factor involved in enzyme production other than the substrate. Bellamy and Gunsalus (10) followed up this suggestion and tested the effect of the addition of various vitamin B factors on the activity of *S. faecalis* growing in a medium consisting of hydrolyzed gelatin, potassium phosphate, glucose, yeast extract, tyrosine, tryptophan, and cystine. Table II shows the effect of adding eight growth

TABLE II

ACTION OF THE PRESENCE OF B VITAMINS IN GROWTH MEDIUM ON THE TYROSINE DECARBOXYLASE ACTIVITY OF *S. faecalis* (10)

Medium	$Q^{N}_{CO_2}$ (tyrosine)
Basal (see text)	77
Basal + eight factors as below	890
Basal + factors without thiamin	650
riboflavin	550
pyridoxine	210
pantothenate	800
nicotinic acid	130
p-aminobenzoic acid	780
biotin	640
"folic acid"	680
Basal + pyridoxine (100 μg./100 ml.) + nicotinic acid (500 μg./100 ml.)	900
Fully nutrient medium	2500

factors to this medium and then of the omission of these factors one at a time. The Q_{CO_2} values are calculated on a nitrogen basis and the values are correspondingly higher than others calculated on a dry weight basis and quoted in this paper. The addition of the eight growth factors results in a marked enhancement of the tyrosine decarboxylase activity, although the activity is still considerably below that recorded for growth in a fully nutrient medium (10). Omission of each of the factors, in turn, shows that all have some effect, possibly within experimental error in some cases, but that the omission of either pyridoxine or nicotinic acid has a marked effect on the activity, while the addition of these two factors together has an enhancing effect equal to that of the complex of eight factors. The development of the full activity in this organism is therefore dependent upon the presence of pyridoxine and nicotinic acid in the growth medium. The addition of these two factors to the simplified medium used for the growth of *E. coli* will not increase the adaptive formation of arginine decarboxylase by that organism.

4. *pH of Growth Medium*

In general, when bacteria are grown in an alkaline medium, they attack amino acids by deamination, and when growth occurs in an acid medium, by decarboxylation (31). This is correlated with a change in the ionization of the amino acid substrate and with a change in the enzymic constitution of the organism with the *p*H of the growth environment. If bacteria are grown in casein digest medium, free from fermentable carbohydrate, adjusted to various *p*H values within the growth range, and the organisms are harvested and their activities tested for amino acid decarboxylases within their potential enzymic constitution, we find that the enzymes are not formed when growth takes place at an alkaline *p*H and that the lower the *p*H, on the acid side, during growth, the greater the formation of the decarboxylases (24, 25, 27).

TABLE III

RELATION BETWEEN AMINO ACID DECARBOXYLASE ACTIVITY AND *p*H OF THE MEDIUM DURING GROWTH*

Decarboxylase	Organism	*p*H of medium during growth				
		8.5	8.0	7.0	6.0	5.0
l(+)-Ornithine	*E. coli*	0	10	80	310	980
	Cl. septique	..	80	220	380	680
l(−)-Tyrosine	*S. faecalis*	0	15	250	530	670
	Cl. aerofoetidum	0	15	38	48	57
l(+)-Lysine	*E. coli*	2	30	118	250	410
	E. coli†	..	..	218	426	721
l(+)-Arginine	*E. coli*	0	3	20	70	120
l(+)-Glutamic acid	*E. coli*	15	18	51	75	89
	Cl. bifermentans	0	15	95	110	130
l(−)-Histidine	*E. coli*	0	0	1	3	33
	Cl. welchii	0	1	4	8	28

* Activities expressed as Q_{CO_2} = μl. CO_2 liberated from substrate per hr. per mg. dry weight of bacteria, measured at 30° C. and at the optimum *p*H in each case.
† Cells disintegrated by drying in acetone, ether, and air.

Table III shows the activity of various decarboxylases of various organisms grown at environmental *p*H values between 8.5 and 5.0. That the activity figures really represent an increase in the enzyme content of the cells with acid growth conditions has been shown in the case of the lysine decarboxylase of *E. coli* by disintegrating the cells by acetone treatment and measuring the activity of the extracts (values marked with †). It is a common finding that such disintegration of the cells results in an increased activity (16, 21, 32). Since the enzymes are formed only during growth in the presence of their substrates and at an acid *p*H, it follows that

the formation of the enzymes is strictly adaptive to the presence of the specific substrate in the form with an unionized —COOH group.

The enzymes are formed to the greatest extent when growth takes place near the limit of acid tolerance for the organism concerned, but the crop formed under such conditions is small. For large-scale work it is more satisfactory to grow the organism in a medium containing glucose or other fermentable carbohydrate; the fermentation of the carbohydrate during growth greatly increases the crop and, at the same time, causes a fall of *p*H, with the result that a heavy crop of active organisms is produced. The presence of the carbohydrate appears to have no effect on activity that cannot be attributed to the change in *p*H resulting from fermentation (24).

5. *Effect of Growth Temperature*

Eggerth (17) noted that the production of histamine by some organisms is greater when growth takes place at low temperatures than at the usual temperature of 37° C. Gale (24) found that the production of amino acid decarboxylases by strains of *E. coli* is inhibited by growth temperatures of the order of 37° and that optimum formation of the enzymes takes place when growth occurs at 20–26°. Table IV shows that the effect is again due to a difference in the enzyme content of the cells since similar differ-

TABLE IV

EFFECT OF GROWTH TEMPERATURE ON THE FORMATION OF AMINO ACID DECARBOXYLASES IN *E. coli**

Decarboxylase	Preparation	Growth temperature		
		20° C.	26° C.	37° C.
l(+)-Arginine	Washed cells	...	240	128
l(−)-Histidine	Washed cells	...	18	12
l(+)-Ornithine	Washed cells	...	90	8
l(+)-Lysine	Washed cells	252	205	55
l(+)-Lysine	Acetone-dried cells	421	337	156

* Activities expressed as Q_{CO_2} = μl. CO_2 liberated per hr. per mg. dry weight of preparation.

ences in activity with growth temperature are obtained whether the activity measurements are made with intact or acetone-dried cells. This temperature effect is characteristic of the organism rather than of the enzyme, for amino acid decarboxylases are formed to a greater extent at 37° than at 25° in organisms such as *S. faecalis* or *Cl. welchii* (25, 27).

6. *Age of Culture*

When growth takes place under optimal conditions the formation of amino acid decarboxylases does not take place until fairly late in the growth period. Cultures harvested within ten hours (at 25° C.) or four hours (at 37° C.) of inoculation have little or no decarboxylase activity whatever the medium *p*H, and the activity develops as growth proceeds, becoming optimal at about the time that active cell division ceases (24). This is illustrated for various decarboxylases in three organisms in Table V.

TABLE V

EFFECT OF AGE OF CULTURE ON DEVELOPMENT OF AMINO ACID DECARBOXYLASES IN GROWING BACTERIA*

Age of culture, hrs.	Decarboxylase substrate				
	Arginine	Lysine	Glutamic acid	Tyrosine	Histidine
4	...	...	..	...	2
6	4	5	6	50	13
8	2	10	5	103	26
10	6	30	4	143	28
12	70	153	27	286	27
14	105	282	50	325	28
16	110	316	75	330	..
18	103	310	73	286	..
Growth ceases at	16 hrs.	16 hrs.	16 hrs.	15 hrs.	10 hrs.
Growth temp., ° C.	25°	25°	25°	37°	37°
Organism	*Escherichia coli*			*S. faecalis*	*Cl. welchii*

* Growth medium = tryptic digest of casein + 2% glucose. Organisms harvested at times indicated and activities determined with washed suspensions at 30° and optimum *p*H. Activities expressed as Q_{CO_2} = μl. CO_2 liberated per hr. per mg. dry weight of organism.

When growth takes place in the presence of carbohydrate, the delay in the appearance of the enzymes within the cells can be partly, but not wholly, explained by the necessity for the development of a suitable degree of acidity in the medium, but similar effects are obtained in any case in the absence of carbohydrate (27).

General.—For cultivation of active organisms on a large scale, growth has been carried out in a casein digest containing 2% glucose (with the addition of 0.1% marmite for growth of streptococci) at 25° for coliform organisms or 37° for streptococci and clostridia, and growth has been continued until active cell division ceases before organisms were harvested.

IV. Preparation of Cell-Free Amino Acid Decarboxylases

With the exception of ornithine decarboxylase, the enzymes are unaffected by acetone drying of the bacterial cells containing them. The

enzymes can then be extracted from the dried cells by incubation with buffer solutions. The lysine, arginine, glutamic acid, and histidine enzymes can be extracted from appropriate preparations with borate buffer at *p*H 8.5 and the tyrosine enzyme can be extracted from streptococcal powders with acetate buffer at *p*H 5.5. The cell debris can be spun off leaving an opalescent liquid containing the active enzyme (21, 22, 32, 65). The enzymes can then be purified by adsorption on to and elution from alumina Cγ [lysine and histidine decarboxylases (22, 32)] or from calcium phosphate [tyrosine enzyme (21)], followed by fractionation with ammonium sulfate solutions as indicated in Table VI. In this way lysine decarboxylase has been purified 90 times to a final activity at 30° of $Q^{C}_{CO_2}$ = 46,000; tyrosine decarboxylase 116 times to $Q^{C}_{CO_2}$ = 46,300; and histidine decarboxylase 52 times to $Q^{C}_{CO_2}$ = 3670.

TABLE VI

METHODS USED FOR THE PARTIAL PURIFICATION OF THE *l*(+)-LYSINE, *l*(−)-TYROSINE, AND *l*(−)-HISTIDINE DECARBOXYLASES (21, 22, 32)

Decarboxylase substrate:	*l*(+)-Lysine		*l*(−)-Tyrosine	*l*(−)-Histidine
Organism used as source:	*E. coli*	*B. cadaveris*	*S. faecalis*	*Cl. welchii* B.W. 21
Treatment	$Q^{C*}_{CO_2}$	$Q^{C}_{CO_2}$	$Q^{C}_{CO_2}$	$Q^{C}_{CO_2}$
(*1*) Washed cell suspension	508	963	400	71
(*2*) Acetone-powder suspension	860	1,830	800	145
(*3*) Crude extract from (*2*)	1,850	2,170	1,690	254
(*4*) Eluate from				
(a) alumina Cγ	4,100	4,050	....	577
(b) calcium phosphate	....	....	5,040	...
(*5*) Precipitated with ammonium sulfate	4,210	4,500	6,220	715
(*6*) First ammonium sulfate fractionation:				
(a) 40–56% satd. $(NH_4)_2SO_4$	21,100	7,650	....	...
(b) 50–63%	....	....	18,400	...
(c) 50–67%	....	....	....	*3,670*
(*7*) Second fractionation (of *6*)				
(a) 40–47% satd. $(NH_4)_2SO_4$	41,300	17,500	....	...
(b) 53–58%	....	....	45,700	...
(*8*) Third fractionation (of *7*)				
41–47% satd. $(NH_4)_2SO_4$	*46,000*	*33,500*	....	...
(*9*) Repetition of stage *4*	....	....	*46,300*	...
Purification achieved	90	35	116	52

* $Q^{C}_{CO_2}$ = μl. CO_2 liberated from substrate per hr. per mg. carbon of preparation. Highest activities obtained in each case indicated by italicized figures.

The ornithine decarboxylase will not stand acetone drying and loses 90–95% of its activity when organisms containing it are so treated. A cell-free preparation of orni-

thine decarboxylase has been achieved by disintegrating a thick active suspension of *Cl. septicum* by shaking with minute glass beads in a machine constructed from the directions given by Curran and Evans (15). After 2 hours of shaking, the majority of the cells are disintegrated and can be centrifuged down leaving the active enzyme in the opalescent supernatant. It has not been possible to purify this enzyme any further because it is very unstable.

One of the objects of purifying the enzymes in a cell-free state is to obtain specific preparations of each. In some cases this can be done by choosing, as the starting material, an organism specific for the decarboxylation of one amino acid only. Thus, *Cl. welchii* S.R.12 decarboxylates *l*(+)-glutamic acid only; *Cl. septicum* Pasteur is specific for *l*(+)-ornithine; *S. faecalis* for *l*(−)-tyrosine; and a strain of *E. coli* has been isolated which is specific for *l*(+)-arginine. *l*(+)-Lysine decarboxylase is found in many coliform organisms in association with other decarboxylases, but the method of purification worked out by Gale and Epps (32) starting with *Bacterium cadaveris* results in a specific enzyme preparation. The organism most active toward *l*(−)-histidine is *Cl. welchii* but the enzyme is accompanied in this organism by glutamic acid decarboxylase; the preparation of *l*(−)-histidine decarboxylase from *Cl. welchii* B.W.21 results in the destruction of the glutamic acid enzyme and the production of a preparation specific for the decarboxylation of histidine (22).

V. Properties of Purified Amino Acid Decarboxylases

1. Specificity

The distribution of the six amino acid decarboxylases among the potential enzymic constitutions of bacteria indicates that six enzymes, each specific for one amino acid, are involved. Six specific preparations have now been worked out (21, 22, 30, 32, 65) and in each case the preparation will catalyze the decarboxylation of a single amino acid only. Thus *l*(+)-lysine decarboxylase will not attack *d*(−)-lysine or α-methyl-, ε-methyl-, α-acetyl-, or ε-acetyl-*l*(+)-lysine (32); *l*(−)-tyrosine decarboxylase will not attack *d*(+)-tyrosine, *l*(−)-phenylalanine, *dl*-serine, *l*-tyrosine sulfonic acid, N-methyl-*dl*-tyrosine, methoxy-*l*-tyrosine, N-methyl-methoxy-*l*-tyrosine, *l*-thyroxine, *l*-thyronine, or glycyltyrosine (21); *l*(−)-histidine decarboxylase will not attack *d*(+)-histidine, β-alanyl-histidine, acetyl- or benzoylhistidine, thiohistidine or any histidine peptide (22, 34); *l*(+)-glutamic acid decarboxylase will not attack *d*(−)-glutamic acid, N-methylglutamic acid, α-ketoglutaric acid, *l*(−)-aspartic acid, or glutathione (30). These examples demonstrate that the enzyme is specific

in each case for the natural isomer of the complete unsubstituted amino acid substrate.

The substitution of a hydroxy group in the substrate molecule, other than in any of the essential polar groups, does not interfere with decarboxylation. Thus *l*(+)-lysine decarboxylase can decarboxylate *l*(+)-hydroxylysine; *l*(−)-tyrosine decarboxylase can decarboxylate *l*-3,4-dihydroxyphenylalanine ("dopa"), and *l*(+)-glutamic acid decarboxylase can decarboxylate synthetic β-hydroxy-*l*-glutamic acid. In each case the rate of decarboxylation of the hydroxy derivative is less than that of the unsubstituted amino acid substrate (21, 22, 32).

The decarboxylation is quantitative and manometric estimation of the carbon dioxide evolved from the substrate in the presence of the specific enzyme can be used for the estimation of the substrate. The method can be applied to the estimation of amino acids in protein hydrolyzates (30). Table VII gives some typical results obtained for the analysis of protein hydrolyzates by the decarboxylase method with results obtained by other methods and workers quoted for comparison. In every case there is agreement within experimental error: these results provide a further demonstration of the specificity of the decarboxylase preparations used.

TABLE VII

SPECIFICITY OF AMINO ACID DECARBOXYLASE PREPARATIONS: ANALYSIS OF PROTEIN HYDROLYZATES (30)*

Amino acid	Protein	Analysis by specific decarboxylase method	Analysis by other methods and references
l(−)-Histidine	Edestin	3.66	3.50 (50)
	Hemoglobin	12.51	12.56 (68)
l(+)-Lysine	Edestin	2.44	2.44 (50)
	Hemoglobin	10.47	9.4 (68)
l(−)-Tyrosine	Edestin	1.83	1.85 (49)
	Hemoglobin	1.41	1.43 (23)
l(+)-Arginine	Edestin	27.5	28.7 (50)
	Hemoglobin	6.84	6.95 (50)
l(+)-Glutamic acid	Edestin	10.06	10.04 (8)
	Hemoglobin	4.42	3.76 (8)
l(+)-Ornithine	Edestin	Nil	Nil . .
	Tyrocidine	13.11	13.2 (36)

* Amino acid contents of protein hydrolyzates expressed as per cent total nitrogen.

2. *pH of Optimum Activity*

The amino acid decarboxylases are active over a narrow range of *p*H with optima in all cases lying between *p*H 2.5 and 6.0. Table VIII lists the *p*H optima determined for the various enzymes as investigated (*a*)

in the intact cell and (*b*) in purified preparations. The *p*H optima found for the activity measured with intact cells are found to vary from culture to culture and with the composition of the growth medium, but in most cases are found to have a value more acid than the corresponding values found with cell-free preparations. Thus the *p*H optimum of *l*(−)-histidine decarboxylase in washed suspensions of *Clostridium welchii* varies between 2.5 and 3.0 (27), but the value determined with the purified preparation is steady at *p*H 4.5 (22); similarly, the *p*H optimum for *l*(+)-arginine decarboxylase in washed suspensions of *Escherichia coli* varies between 4.0 and 4.8, while the steady value for the cell-free preparation is 5.2 (65). Decarboxylation at an acid *p*H results in the formation of an alkaline amine and the evolution of carbon dioxide with a consequent shift of the environmental *p*H toward neutrality. It is suggested later that one of the functions of the amino acid decarboxylases is to act as a neutralization mechanism in an unfavorably acid medium, and it may be that this difference between the *p*H optima of the enzymes as measured in intact cells and in cell-free preparations is some reflection of the difference between the *p*H values of the internal and external environment of the cell as a result of the decarboxylase action.

3. *Effect of Substrate Concentration*

Table VIII also lists the Michaelis constants of the cell-free preparations compared with the apparent Michaelis constants measured with washed

TABLE VIII

PROPERTIES OF THE AMINO ACID DECARBOXYLASES

Decarboxylase	*p*H optimum of		Michaelis constant of	
	Intact cell preparation	Cell-free preparation	Intact cell preparation	Cell-free preparation
l(−)-Histidine	2.5–3.0	4.5	0.00075*M*	0.00075*M*
l(+)-Lysine	4.5–5.0	6.0	0.0028	0.0015
l(+)-Arginine	4.0–4.8	5.2	0.00056	0.00075
l(+)-Glutamic acid	4.0–4.5	4.5	0.005	0.027
l(+)-Ornithine	5.0–5.5	5.2	0.003	0.004
l(−)-Tyrosine	5.0–5.5	5.5	.....	.....

suspensions of organisms as source of enzyme. The affinities of the enzymes differ widely from that of *l*(+)-arginine decarboxylase (Michaelis constant = 0.00075 *M*) to that of *l*(+)-glutamic acid decarboxylase (Michaelis constant = 0.027 *M*) and only in the latter case is there any marked difference between the affinity of cell-free preparation and of the intact

organism preparation of the enzyme. In the case of *l*(+)-glutamic acid decarboxylase there is a marked decrease in the affinity following extraction of the enzyme from the cell, suggesting that the enzyme undergoes some damage during the preparative process. It is not possible to obtain values for the Michaelis constant of the tyrosine enzyme as the enzyme is not saturated by saturated solutions of tyrosine.

4. Quantitative Nature of the Decarboxylation

Estimation of the carbon dioxide evolved during the decarboxylation of the six amino acids by the six decarboxylase preparations shows that, when allowance is made for carbon dioxide retention, gas output represents 96–98% theoretical (30). The quantitative nature of the decarboxylation is confirmed by the analytical figures quoted above in Table VII. If racemic mixtures of the amino acid substrate are used, then the carbon dioxide output corresponds to 45–49% theoretical (21, 22, 32) and the preparations can be used as a method for the resolution of such racemic mixtures and the preparation of the unnatural isomer (52).

5. Action of Inhibitors

Table IX summarizes the action of common inhibitors on the action of the six amino acid decarboxylases (65). The enzymes are all sensitive to the presence of *silver* ions, the tyrosine and glutamic acid enzymes being considerably less sensitive than the others. All the enzymes are sensitive to *mercury* ions, the tyrosine enzyme again being the least sensitive. With the exception of the ornithine decarboxylase, which is completely inhibited by $M \times 10^{-5}$ copper, the decarboxylases are not abnormally sensitive to the presence of *copper* ions and none has any marked sensitivity to *iron* ions. All the enzymes are inactivated by *potassium permanganate*, ornithine decarboxylase being particularly sensitive, a fact which may be correlated with the sensitivity of this enzyme to copper.

All six decarboxylases are sensitive to *cyanide* and it has been shown in the case of the lysine and arginine enzymes that this inhibition is reversible (32). This suggests the presence of a metal in the enzyme constitution although the enzymes, other than lysine decarboxylase, are not markedly sensitive to *sodium azide*. Lysine decarboxylase is 95% inhibited by 0.001 *M* hydrocyanic acid or sodium azide, but is not affected by carbon monoxide, hydrogen sulfide, sulfanilamide, or the "copper inhibitors" (32, 64); spectrometric examination of ashed purified enzyme preparations failed to show the presence of any metals in significant amounts (32).

The cyanide sensitivity may be due to the presence of an aldehyde group in the constitution of some of the enzymes (see below).

The significance of the sensitivity of some of the enzymes to keto fixatives such as *hydroxylamine*, *hydrazine*, and *semicarbazide* will be discussed below.

TABLE IX

EFFECT OF INHIBITORS ON ACTIVITY OF CELL-FREE AMINO ACID DECARBOXYLASES (65)*

Inhibitor	pI	Amino acid decarboxylase					
		Lysine	Tyrosine	Arginine	Ornithine	Histidine	Glutamic acid
$AgNO_3$	5	98	...	...	98	13	4
	4	100	...	35	100	100	20
	3	100	39	100	100	100	81
	2	100	99	100	100	100	100
$HgCl_2$	5	98	...	14	...	...	...
	4	100	...	100	35	58	27
	3	100	...	100	100	94	100
	2	100	97	100	100	100	100
$CuSO_4$	6	...	...	...	27	...	...
	5	...	...	...	100	...	...
	4	46	...	7	100	...	4
	3	89	98	51	100	61	10
	2	98	100	100	100	92	33
$FeSO_4$	4	30	...	...	16	...	...
	3	82	26	23	32	...	0
	2	100	66	54	67	15	0
$KMnO_4$	5	10	24	...	53	...	6
	4	100	100	17	98	15	41
	3	100	100	100	100	94	100
KCN	4	81	...	22	40	...	93
	3	93	64	98	77	20	100
	2	100	93	100	96	97	100
NaN_3	4	61	...	7	36	...	...
	3	98	...	21	51	...	10
	2	100	0	100	97	0	27
$NH_2.NH_2$	5	95	...	62	80	...	...
	4	100	30	71	92	...	0
	3	100	98	95	100	...	13
	2	100	100	100	100	10	43
NH_2OH	5	95	43	91	50	...	7
	4	100	97	95	98	54	42
	3	100	100	100	100	100	96
Semicarbazide	6	42	...	...	...	0	...
	5	76	90	45	77	33	0
	4	98	98	78	97	52	3
	3	100	100	94	100	77	6
	2	100	100	100	100	89	24
Sulfanilamide	2	15	9	21	35	51	0
Codecarboxylase present in enzyme		+	+	+	+	−	−

* pI = negative logarithm of molar concentration of inhibitor. Inhibition expressed as percentage.

Sulfanilamide has a marked effect on histidine decarboxylase and this inhibition appears to be specific for the complete sulfanilamide molecule as neither N^4- nor N^1-substituted sulfonamides are active as inhibitors (22).

Certain of the enzymes, particularly the ornithine and lysine enzymes, are inhibited by the presence of high concentrations of salts such as ammonium sulfate or sodium sulfate (32, 65). The effect is reversible and is not apparently related to a coenzyme dissociation (65).

Common inhibitors which have no significant effect on the decarboxylases in concentrations less than 0.01 *M* are iodoacetate, fluoride, urethan, chloramine T, 8-hydroxyquinoline, sulfathiazole, etc.

VI. Resolution of Enzymes into Apoenzymes and Coenzyme Moieties

Precipitation of *l*(+)-lysine decarboxylase with ammonium sulfate at an alkaline *p*H yields a protein which, on solution, is inactive toward lysine but which can be reactivated by the addition of boiled preparations of enzyme, bacteria, yeast, liver, etc. This suggests that the lysine decarboxylase dissociates at an alkaline *p*H into specific protein apoenzyme and coenzyme moieties with the result that precipitation of the protein with ammonium sulfate results in a coenzyme-free apoenzyme preparation. It is not possible to prepare the apoenzyme by dialysis against distilled water or ammonium sulfate solutions, as such treatment results in inactivation of the protein. The most satisfactory method of preparation is to precipitate the enzyme twice with 66% saturated ammonium sulfate solution containing 10% by volume of 0.880 ammonia solution. Table X shows that the protein so precipitated can be reactivated toward lysine by the addition of coenzyme (codecarboxylase) prepared as described below. The double precipitation with ammoniacal ammonium sulfate does not always result in complete resolution of the enzyme but this occurs if the preparation is kept in solution for a few days in the ice chest (32).

The *l*(−)-tyrosine decarboxylase spontaneously dissociates to a certain extent during purification (21) and consequently must be tested in the presence of excess coenzyme for activity determinations. The dissociation can be completed either by two consecutive precipitations with 66% saturated ammonium sulfate containing 3% by volume of 0.880 ammonia, or by standing the enzyme solution in the ice chest for 72 hours followed by dialysis overnight against glass-distilled water. In either case the resulting preparation is activated toward tyrosine by the addition of codecarboxylase preparations.

The precipitation of the lysine and tyrosine decarboxylases by am-

moniacal ammonium sulfate results in the irreversible inactivation of some of the enzyme—amounting to about 50% in the case of the tyrosine enzyme and 60–70% of the lysine enzyme. Preparations of the arginine decarboxylase are more difficult to resolve in this manner but an arginine apodecarboxylase preparation has been made by repeated precipitation of the protein with ammonium sulfate solutions containing progressively larger amounts of ammonia solution (65). The preparation was activated toward arginine by the addition of codecarboxylase preparations. Ornithine decarboxylase is irreversibly inactivated by ammonium sulfate precipitation but solutions of the enzyme dissociate spontaneously on standing; the dissociation is presumably followed by decomposition (probably oxidation) of the coenzyme as the activity of the preparations rapidly decreases on standing, but can be restored by the addition of codecarboxylase preparations (65).

The apoenzyme preparations of any one of these four enzymes can be activated toward their substrates by the addition of a boiled preparation of any one of the untreated enzymes, thus indicating that the four enzyme preparations contain a substance or substances that will function as coenzyme or coenzymes to the four apoenzymes.

It has not been possible to resolve or prepare apoenzymes of either the histidine or the glutamic acid decarboxylases.* In both cases the enzyme preparations have been submitted to precipitation by ammoniacal ammonium sulfate of various strengths, to dialysis against distilled water or ammonium sulfate solutions, to prolonged standing at 0° C., to precipitation at acid *p*H values, etc., and in no case has any reversible resolution been achieved (22, 65). Further, boiled preparations of either histidine decarboxylase or glutamic decarboxylase will not act as a source of coenzyme for tyrosine or lysine apodecarboxylases. This suggests that these two enzymes differ from the remaining four decarboxylases in that they do not possess codecarboxylase as part of the enzyme structure (22, 65).

In Table IX the enzymes are grouped according to whether they have been demonstrated to contain codecarboxylase or not. The survey of the action of inhibitors was undertaken in order to find out whether it is possible to differentiate between codecarboxylase enzymes and noncodecarboxylase enzymes by sensitivity to inhibitors (65). Inspection of Table IX shows that two groups of inhibitors differentiate between the two types of decarboxylase. First, although none of the enzymes is markedly sensitive

* Umbreit and Gunsalus (65a) claim to have effected a partial resolution of glutamic decarboxylase from a strain of *E. coli* by dialysis for 24 hrs. at *p*H 2 and 0° C. The activity of the preparation was increased by addition of either pyridoxal phosphate or codecarboxylase concentrate.

TABLE X

REVERSIBLE RESOLUTION OF AMINO ACID DECARBOXYLASES

Decarboxylase	Treatment	μl. CO_2 liberated per 5 min.	
		Alone	Plus codecarboxylase
l(+)-Lysine	Twice pptd. with 66% satd. ammonium sulfate containing 10% ammonia (sp. gr. = 0.880)	20	195
l(−)-Tyrosine	Dialysis against glass-distd. water	10	174
l(+)-Arginine	As for lysine enzyme	20	118
l(+)-Ornithine	Standing 48 hrs. at 0° C.	25	110
l(−)-Histidine	No resolution achieved	..	...
l(+)-Glutamic acid	No resolution achieved	..	...

to *iron*, the four codecarboxylase enzymes are markedly inhibited by 0.01 *M* Fe^{++}, which has no significant inhibitory action on either the histidine or the glutamic acid decarboxylase. Second, the group of keto fixatives are all more inhibitory toward the codecarboxylase enzymes than toward the other two enzymes. *Hydrazine* in a concentration of 0.001 *M* produces 95–100% inhibition of the codecarboxylase enzymes, but no inhibition of histidine decarboxylase (which is not completely inhibited by 0.1 *M* hydrazine), and 13% inhibition of the glutamic acid enzyme. *Hydroxylamine* in 0.0001 *M* concentration produces 95–100% inhibition of the codecarboxylase enzymes, 54% inhibition of histidine decarboxylase, and 42% inhibition of glutamic acid decarboxylase. *Semicarbazide* in 0.001 *M* concentration produces insignificant inhibition of the glutamic acid enzyme, 77% inhibition of the histidine enzyme, but 94–100% inhibition of the other four enzymes which, with the exception of the arginine enzyme, are 97% inhibited by 0.0001 *M* semicarbazide. These results indicate that the codecarboxylase enzymes possess a keto or aldehyde group which is essential for enzymic activity.

VII. Distribution, Properties, and Preparation of Codecarboxylase

1. Distribution

The apoenzyme preparations of the lysine, tyrosine, arginine, and ornithine decarboxylases are activated toward their substrates by the addition of a source of codecarboxylase to the reaction mixture. Consequently, the apoenzymes can be used for a test for the presence of codecarboxylase since, if a suitable preparation of any substance will produce an activation

of the apoenzyme toward its substrate, it is highly probable that that substance contains codecarboxylase or a closely related product. Further, since the rate of decarboxylation of substrate is roughly proportional to the amount of codecarboxylase added to a given amount of apoenzyme, as long as the enzyme is not saturated with coenzyme (see Fig. 1, page 27), it is possible to assay the codecarboxylase content of tissues, etc., on a comparative basis. This has been done with a number of cells using *l*(+)-lysine apodecarboxylase and adding amounts of boiled, disintegrated cells

TABLE XI

DISTRIBUTION OF CODECARBOXYLASE* (33)

Tissue	Units codecarboxylase per mg. C
Rat skeletal muscle	1.54†
Bacillus subtilis	1.5
Staphylococcus aureus	1.48
Rat liver	1.22
Escherichia coli	1.12
Brewers' yeast	1.03
Pseudomonas aeruginosa	0.765
Rat kidney	0.63
Clostridium welchii	0.465
Sarcina lutea	0.41
Rat heart	0.385†
Rat lung	0.38
Saccharomyces cerevisiae	0.34
Brassica oleracea (wild cabbage)	0.29†
Pisum sativum (garden pea)	0.24†
Rat gut	0.23†
Rat brain	0.21
Streptococcus faecalis	0.13
Cow milk	0.043
Rat blood	0.037†
Human urine	Nil

* Codecarboxylase content expressed as units of lysine codecarboxylase per mg. carbon of tissue (33).

† Sampling errors make these analyses approximate only.

so that, for a given amount of enzyme, the amount of codecarboxylase added in each case was never more than sufficient to produce a rate of decarboxylation half that of the saturated enzyme (33). Table XI gives the results found for various cells and quoted as units of lysine codecarboxylase per milligram carbon of tissue; the figures have no absolute values but serve to show the comparative codecarboxylase contents of the tissues. It is obvious that codecarboxylase has a wide distribution, being found in all the living cells investigated whether of bacteria (irrespective of amine

production), yeasts, or animal or plant tissues. If the tissues are assayed with regard to the codecarboxylase of tyrosine apodecarboxylase it is found, with one or two insignificant exceptions, that the distribution of the codecarboxylase is qualitatively and quantitatively the same whichever apoenzyme is used for assay purposes.

The following substances have been tested for codecarboxylase activity with negative results: coenzyme I, coenzyme II, thiamin, thiamin monophosphate, thiamin diphosphate, riboflavin, riboflavin phosphate, riboflavin-adenine-dinucleotide, adenylic acid, adenine, adenosine, inosine, hypoxanthine, glutathione, yeast nucleic acid, nicotinic acid, nicotinic amide, uracil, biotin, pantothenic acid, pyridoxine, *p*-aminobenzoic acid, inositol, thiochrome, xanthopterin, "folic acid," pyridoxylic acid, and various mixtures of these substances.

2. Properties

Using apoenzyme preparations as a method of assaying codecarboxylase, it is possible to investigate the properties of the coenzyme. For these studies a codecarboxylase preparation worked up from dried brewers' yeast has been used (33).

Salts.—Codecarboxylase forms salts with metals. The *silver*, *mercury*, and *lead* salts are soluble in acid and precipitated at neutrality; the active substance cannot be regenerated from these precipitates by hydrogen sulfide as the codecarboxylase then becomes adsorbed on the metallic sulfides and no satisfactory method of elution has been discovered. The *barium* salt is soluble in water at all *p*H values tested but is precipitated by 60% ethanol or methanol at *p*H 7.0. Codecarboxylase is precipitated by *phosphotungstic acid*, but not by *picric* or *picrolonic* acids.

Solubility in Organic Solvents.—Codecarboxylase cannot be extracted from acid, neutral, or alkaline solutions into ether; it can be extracted from acid solution into *n*-butanol, isobutanol, phenol, or cresols, but not from alkaline solution; the free acid is soluble in ethanol or methanol.

Stabilities.—Purified codecarboxylase preparations are rapidly inactivated by boiling in weak acid, two hours in 0.1 *N* sulfuric acid at 100° C. resulting in 90% inactivation; on the other hand, they are remarkably stable to alkali, no inactivation being produced by four hours in normal or 0.1 *N* sodium hydroxide at 100°. Purified preparations are unstable in air at *p*H 7.0 undergoing oxidation or hydrolysis at room temperature. Treatment of codecarboxylase with nitrous acid, hypobromous acid, or benzoyl chloride has no effect on activity.

3. *Preparation of Codecarboxylase Concentrate*

A method of concentration of natural codecarboxylase has been worked out on the basis of these properties (33). Convenient starting material is dried yeast (brewers' or bakers') which is extracted at 37° C. with 0.75% baryta and the sediment then centrifuged down, leaving the bulk of the active material in the supernatant fluid. The centrifugate is then purified as in Scheme 2.

	DEGREE OF PURIFICATION
Dried yeast	1
↓ Extract with baryta at room temperature	
Centrifugate	2
↓ Precipitate with two volumes methanol at *p*H 7.0	
Filtrate	
↓ Precipitate with barium acetate	
Precipitate	
Wash exhaustively with hot water	
Decompose in normal sulfuric acid	75
↓ Extract with liquid phenol	
Mother liquor	
Saturate with ammonium sulfate	
↓ Extract with liquid phenol	
Phenol extract	
↓ Extract with water and ether mixture	
Water phase	255
Acidify to *p*H 1 with nitric acid	
↓ Extract with isobutanol	
Isobutanol extract	
↓ Extract with sodium hydroxide	
Alkaline extract	
Neutralize with nitric acid	675
↓ Add lead acetate	
Lead precipitate	
Decompose in sulfuric acid	2,500
↓ Neutralize with excess barium acetate	
Centrifugate	
↓ Add one volume ethanol	
Barium precipitate	4,300
Decompose in sulfuric acid	
↓ Add lead acetate to *p*H 5	
Centrifugate	
Add lead acetate in excess	
↓ Neutralize with dilute sodium hydroxide	15,000
LEAD PRECIPITATE	

SCHEME 2

The final lead precipitate can be dried *in vacuo* and kept indefinitely, being regenerated for use by decomposition in dilute sulfuric acid. It is a yellow-white amorphous powder which gives a faintly yellow solution on decomposition. The composition of the lead salt is not constant and preparations vary considerably in their activities. The stage of the preparation which is least satisfactory is the phenol extraction after saturation of the mother liquor with ammonium sulfate and attempts to increase the scale of the preparation above laboratory scale have been found to result in less active preparations, mainly as a result of inactivation occurring at this stage. Elementary analysis of one

lead salt preparation gave: carbon, 16.0; hydrogen, 2.3; nitrogen, 3.6; phosphorus, 1.0, and lead, 55.5%.

The preparation, freed from lead, is highly active as codecarboxylase for the apoenzymes of lysine, arginine, ornithine, or tyrosine decarboxylases. During preparation the degree of purification achieved at each stage was assayed against both the lysine and the tyrosine enzymes (33) and the increase in purity over each step was the same, within experimental error, whichever enzyme was used for the assay. There is, therefore, little doubt that these two enzymes have the same coenzyme.

The rate of decarboxylation of substrate by the apoenzyme is dependent upon the concentration of codecarboxylase present in the reaction mixture and the curves relating rate of decarboxylation to codecarboxylase concentration are, in all cases, typical of those obtained for an apoenzyme–coenzyme dissociation (see Fig. 1, page 27). In the case of *l*(+)-lysine decarboxylase, the enzyme is half-saturated with coenzyme when an amount of the purified concentrate containing 0.06 μg. carbon per ml. is added to the system (21, 32, 65).

It is certain that the lead concentrate prepared as above is not a pure preparation of codecarboxylase. It will be shown below that the coenzyme is probably pyridoxal phosphate, the lead salt of which would contain 7.4% phosphorus so that the concentrate may not contain more than about 13% codecarboxylase. Attempts to isolate an aldehyde derivative from the lead salt have so far yielded a small amount of a 2,4-dinitrophenylsemicarbazone, which gives a positive test for the presence of phosphate.

VIII. Chemical Nature of Codecarboxylase

Tyrosine Decarboxylase Activity of Pyridoxine-Deficient Streptococci.—Bellamy and Gunsalus (10) showed that, when a strain of *Streptococcus faecalis* with potential tyrosine decarboxylase activity is grown in a simplified medium (page 8), growth takes place, but the resulting organisms have negligible tyrosine decarboxylase activity. They found that the organisms produce the enzyme only if the growth medium contains, in addition to tyrosine, sufficient amounts of pyridoxine and nicotinic acid. Since the organism is nutritionally exacting toward pyridoxine, it follows that the development of the enzyme requires amounts of pyridoxine in excess of simple growth requirements. Snell and others have shown that there is a factor in animal tissues which will replace pyridoxine in the nutrition of certain lactic bacteria and when assayed against *S. lactis* R is more effective than pyridoxine itself; this factor has

been called "pseudopyridoxine" (61). If pyridoxine is treated in various ways as, for example, autoclaving with cystine (56), treatment with hydrogen peroxide (14), etc., its activity increases as a growth factor for *S. lactis* R, suggesting that pseudopyridoxine may be an oxidation product of pyridoxine. *S. lactis* R, which is apparently a strain of *S. faecalis* (40), will grow in a medium in which pyridoxine is replaced by alanine (60) and the cells are then inactive toward tyrosine but can be activated by the addition of pseudopyridoxine preparations made from pyridoxine, the stimulation of the tyrosine decarboxylase system by the preparations being in proportion to their pseudopyridoxine content (11). This presumably means that when the streptococci are grown in a pyridoxine-deficient medium, they are able to synthesize the protein moiety of tyrosine decarboxylase, but not the coenzyme or prosthetic group moiety.

Nature of "Pseudopyridoxine."—Following the indications obtained from microbiological assay concerning the nature of pseudopyridoxine, Harris, Heyl, and Folkers (43) synthesized certain pyridoxine derivatives including pyridoxal (with an aldehyde group in position 4) and pyridoxamine (with an amine group in position 4). Both these derivatives are active as the pseudopyridoxine growth factor for *S. lactis* R (57). Snell (62) finds that pyridoxal has 5000–8000 times, and pyridoxamine 6000–9000 times the activity of pyridoxine as growth factor for *S. lactis* R although the comparative activities are less for other organisms, and all three compounds have approximately the same activity as growth factors for *Saccharomyces cerevisiae*; Snell suggests that the biologically active substance is either pyridoxal or pyridoxamine and that these figures result from the varying ability of organisms to synthesize the aldehyde or amine derivative from pyridoxine. He has produced indirect evidence that pyridoxine, pyridoxal, and pyridoxamine all exist naturally in varying amounts in various tissues (58).

Pyridoxal as Missing Factor in Tyrosine Decarboxylase Activity of Pyridoxine-Deficient Streptococci.—Once the pyridoxine derivatives were available, they were tested for their ability to activate pyridoxine-deficient streptococci toward the decarboxylation of tyrosine (38); washed suspensions of such organisms were activated by pyridoxal but not by pyridoxamine or pyridoxine itself. Using the washed suspensions of streptococci as apoenzyme preparation, the half-saturation concentration of pyridoxal corresponds to 0.15 μg. per 3 ml. giving an apparent dissociation constant for the enzyme–coenzyme complex $= 3 \times 10^{-7}$ molecules per liter. However if the cells are dried, then pyridoxal is no longer effective as activator unless adenosine triphosphate is also added or, alterna-

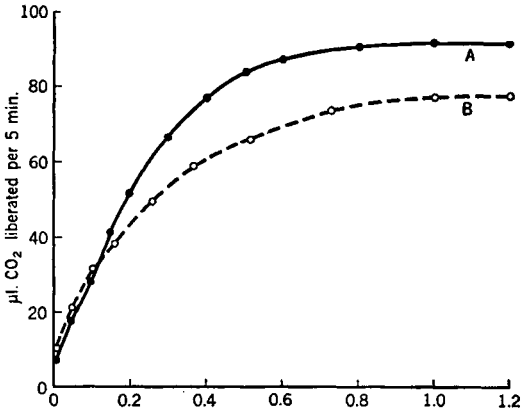
tively, the pyridoxal is phosphorylated by treatment first with thionyl chloride and then by silver dihydrogen phosphate (39).

Codecarboxylase Function of Pyridoxal Phosphate.—The results obtained by Bellamy and Gunsalus suggest strongly that a phosphorylated

TABLE XII
CODECARBOXYLASE ACTIVITY OF "PYRIDOXAL PHOSPHATE" (7)

Apodecarboxylase	μl. CO_2 liberated from substrate per 5 min.		
	Alone	Plus codecarboxylase	Plus pyridoxal phosphate
$l(-)$-Tyrosine	2	110	116
$l(+)$-Lysine	15	106	104
$l(+)$-Arginine	15	70	75
$l(+)$-Ornithine	29	78	74

derivative of pyridoxal is the coenzyme of tyrosine decarboxylase. This has been confirmed by Baddiley and Gale (7) with the apoenzyme preparation of tyrosine decarboxylase. Table XII shows the activation of the



(A) μg. carbon of codecarboxylase preparation per 3 ml.
(B) ml. pyridoxal phosphate preparation per 3 ml., ×10.

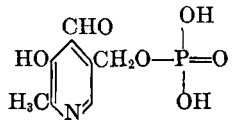
Fig. 1.—Rate of decarboxylation of $l(+)$-lysine by the apoenzyme of $l(+)$-lysine decarboxylase in the presence of codecarboxylase and "pyridoxal phosphate" preparations. Contents of cups: 0.25 ml. apoenzyme solution (preparation A or B); 0.5 ml. $M/15$ $l(+)$ lysine; 2.0 ml. $M/5$ phosphate buffer, pH 6.0; codecarboxylase or "pyridoxal phosphate" preparation as above + water to 0.25 ml. Temperature, 30° C.

apoenzymes of the lysine, tyrosine, arginine, and ornithine decarboxylases toward their substrates by the addition of excess of either codecarboxylase

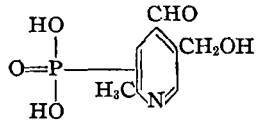
concentrate or of a phosphorylated pyridoxal preparation. Figure 1 shows the variation of the rate of decarboxylation of lysine in the presence of lysine apodecarboxylase and increasing amounts of codecarboxylase concentrate on the one hand and of "pyridoxal phosphate" on the other.

Identity of Codecarboxylase and "Pyridoxal Phosphate."—Up to the time of writing neither the natural codecarboxylase from yeast nor the synthetic pyridoxal phosphate has been obtained in a pure state.* The structure of the active substance in pyridoxal phosphate has not been satisfactorily determined, since both groups of workers have used preparations consisting of pyridoxal treated with thionyl chloride followed by silver dihydrogen phosphate. The evidence suggests that the active material consists of an orthophosphate of pyridoxal in which the phosphate group is on the primary hydroxyl group (as in I):

(I)



(II)



but it is possible that the phosphoryl residue is on the phenolic group (as in II). The properties of the active material in this synthetic preparation are similar to those determined for the natural codecarboxylase, and Table XIII shows that the stabilities of the two active substances toward acid and alkali are also similar. The presence of the aldehyde

TABLE XIII

STABILITIES OF CODECARBOXYLASE AND PYRIDOXAL PHOSPHATE (7)†

Treatment	Codecarboxylase, units per ml.	Pyridoxal phosphate, units per ml.
Initial untreated	32	23
After 1 hr. at 100° C. in		
0.1 *N* NaOH	31	24
N NaOH	31	20
0.1 *N* H_2SO_4	11	7

† Activity estimated against tyrosine apodecarboxylase and expressed as units codecarboxylase per ml. of preparation in each case.

* Gunsalus, Umbreit, Bellamy, and Foust (40a) describe the preparation of a purified synthetic product as its barium salt. The preparation contains 6.2% organic phosphorus, about 50% barium, and 32% pyridoxal estimated from the ultraviolet spectrum; the phosphorus content thus corresponds to one phosphate radical per mole of pyridoxal, which is in agreement with the above formula. Further data in this paper suggest that the phosphoryl residue in the synthetic product is on the phenolic group and not on the primary hydroxyl group.