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The Effects of Phosphates, Arsenates and Nucleotides on L-Amino Acid Decarboxylases

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Gale & Epps (1944) and Gale (1945) observed that the lysine decarboxylase activity of *Escherichia coli* '86' was increased by inorganic phosphate (0·1–0·5 M), and that the decarboxylation of ornithine by *Clostridium septicum* was more rapid in phosphate buffers than in citrate buffers of comparable pH. Krebs, Eggleston & Knivett (1955) recently confirmed that the decarboxylation of L-ornithine and L-lysine by *Esch. coli* was accelerated by the addition of phosphate at pH 6·5–6·8. The present paper is concerned with a more detailed study of the effects of inorganic and organic phosphates and related substances on the activity of amino acid decarboxylases.

EXPERIMENTAL

Materials

Buffers. The following buffers were used: phosphate, made from molar stock solutions of Na_2HPO_4 and NaH_2PO_4; arsenate, made from 0·5 M-Na_2HAsO_4 and N-HCl; citrate made from 0·2 M sodium citrate and N-HCl or N-NaOH; tris, made from M aminotrishydroxymethylmethane and N-HCl; acetate, made from sodium acetate and acetic acid; acetate–veronal (Michaelis, 1931), and tris–maleate (Gomori, 1955). A glass electrode and a Pye pH meter were used to measure the pH of these buffers after dilution to the concentrations used in experiments. The additions of enzyme and substrates had little or no effect on the pH.

Nucleotides. Adenosine triphosphate (ATP) was prepared in this laboratory by Mr R. Hems. The barium salt from rabbit muscle (LePage, 1949) was dissolved in 0·1 N-HCl and passed through a column of Amberlite resin IR-120 (H) in the H^+ form. The free acid was neutralized with NaOH and concentrated by freeze-drying. Analysis by the method of Krebs & Hems (1953) showed it to be at least 98% ATP, with a trace of adenosine diphosphate (ADP), and no other detectable phosphate impurity. In some experiments two commercial samples of ATP were used; analysis showed them to be mixtures of ATP and ADP (about equal quantities) with about 15% of other impurities including inorganic phosphate, adenosine 5′-phosphate (AMP-5′) and some other unidentified organic phosphates.

Barium ADP was obtained from Schwarz Laboratories Inc. It was found to contain less than 5% ATP.

Muscle adenylic acid (AMP-5′) from Roche Products Ltd., and yeast adenylic acid from L. Light and Co. Ltd., were both found to be 98–100% pure when examined by the chromatographic techniques of Krebs & Hems (1953).

Adenosine 2′-phosphate (AMP-2′) and adenosine 3′-phosphate (AMP-3′) were obtained from L. Light and Co. Ltd. Examination of these compounds by the chromatographic method of Carter (1950) showed no phosphate impurities. Guanylic, uridylic and cytidylic acids, which are known to be mixtures of the 2′- and 3′-phosphates, were also obtained from L. Light and Co. Ltd.

Inosine triphosphate and inosine 5′-phosphate from Sigma Chemical Co. showed no phosphate impurities when tested by the method of Krebs & Hems (1953).

Flavin mononucleotide was a gift from the Sigma Chemical Co.

Other compounds. Barium ribose 5-phosphate was prepared according to Long (1955). Chromatographic analysis according to Eggleston (1954) revealed traces of AMP-5′, but no other impurity. For use it was dissolved in dilute HCl and a calculated amount of Na_2SO_4 was added to precipitate the barium; after centrifuging, the supernatant solution was adjusted to pH 6·8 with NaOH. Glucose 1-phosphate, glucose 6-phosphate and sodium arsonoacetate ($Na_2O_3.AsCH_2.CO_2Na$) were prepared by Mr D. H. Williamson. Other organic arsenicals were obtained from British Drug Houses Ltd. Organophosphorus insecticides were obtained

from Dr W. N. Aldridge and from Albright and Wilson Ltd. These included: di*iso*propylphosphorofluoridate, diethyl *p*-nitrophenyl phosphate (E 600; paraoxon), octamethylpyrophosphoramide (schradan, OMPA), dimethyl *p*-nitrophenyl phosphorothioate (parathion), tetraethyl pyrophosphate and dimethyldichlorovinyl phosphate. Carbamyl phosphate was prepared by Dr M. Shilo according to Jones, Spector & Lipmann (1955). Pyridoxal phosphate was supplied by Merck and Co. Inc., Rahway, New Jersey, U.S.A.

Micro-organisms. Clostridium welchii S.R. 12 and seven strains of *Escherichia coli* were examined. These included: *Esch. coli* 'F' (*Bact. coli*), a laboratory strain from the Middlesex Hospital, used by Fildes (1940) and by Woods (1940); *Esch. coli* 'T', a laboratory strain isolated in Professor D. D. Woods's laboratory; *Esch. coli* '4', a laboratory strain originating from Department of Biochemistry, University of Sydney, Australia, used by Lascelles & Still (1946); *Esch. coli* var. *communior*, N.C.T.C. no. 86; *Esch. coli* N.C.I.B. no. 8114, American Type culture collection no. 9723; *Esch. coli* 'M/48', origin unknown. All the above were supplied by Professor D. D. Woods. Dr V. A. Knivett supplied *Esch. coli* N.C.I.B. no. 8571 (Knivett, 1954).

Methods

Cl. welchii was grown as described by Krebs (1948). The *Esch. coli* strains were grown in 1 l. spherical, flat-bottomed flasks filled to the neck with a liquid medium containing 1% Lab-lemco (Oxo Ltd.), 0·5% yeast extract (Difco), 0·5% NaCl, 2% Tryptone (a pancreatic digest of casein, Oxo Ltd.) and 2% glucose (sterilized separately), all w/v. After inoculating the flasks with subcultures which had been grown overnight, the flasks were incubated at 27° for 24 hr. During this time the pH fell from about 7 to about 5. The cells were collected by centrifuging, washed twice with water, and stored at 0° or −15° as suspensions in water containing about 30 mg. dry wt. of cells/ml. Cell-free extracts were prepared in the Hughes press (Hughes, 1951) at −25° to −15°, or by shaking 10 ml. suspensions of whole cells with 10 ml. of Ballotini beads no. 14 (Chance Bros., Birmingham) in a Mickle disintegrator (Mickle, 1948) at 50 cyc./sec. for 30 min. at 0°, followed by centrifuging and washing of the sediment with small volumes of water. These extracts were in some instances dialysed against distilled water at 2° overnight, or were re-centrifuged in a high-speed head at 16 300 *g.* to obtain enzymes in clear solution. Acetone–ether dried powders were prepared according to Gale & Epps (1944).

Extracts were made of carrot, vegetable marrow and melons by using a 'Braun' separator (Max Braun Commercial, Frankfurt a. M., Germany). In this instrument the tissue is grated and the liquid fraction is separated from insoluble particles by centrifugal forces. The extracts were filtered through a Jena sintered-glass funnel (grade 3G3) without suction (see Schales, Mims & Schales, 1946). The filtrate was stored at −15°.

Brains from pigeons and rats, and rat livers, were ground with 1 or 2 vol. of water in a mortar, or were homogenized in a stainless-steel Potter–Elvehjem homogenizer. Fresh suspensions were examined immediately wherever possible, as they lost 40–50% of their decarboxylating activity when stored for 1 day at −15°.

The decarboxylation of L-amino acids was examined in Warburg manometers in an atmosphere of N_2. Preliminary experiments (on the bacterial decarboxylation of arginine) were carried out at 40°, but as the bacterial decarboxylases are known to be rapidly inactivated at this temperature (Gale, 1940) most of the subsequent work on bacterial and plant enzymes was done at 25°. In experiments with animal tissues the temperature was 35°.

In experiments buffered at pH 6·3–6·8 a correction was made for the bound CO_2 according to Johnson (1951). Above pH 6·8 this procedure is not sufficiently accurate and in this pH range Warburg vessels with two side arms were used for the determination of the total CO_2 production, substrate being placed in one side arm, 0·5 ml. of 10% (v/v) H_2SO_4 in the other. The addition of the acid at the end of the experiment gave the value for bound CO_2. In some experiments the results are expressed as Q_{CO_2} (μl. of CO_2/mg. dry wt. of enzyme/hr.).

Paper chromatography of nucleotides was carried out on trichloroacetic acid filtrates according to Krebs & Hems (1953) and Carter (1950). For the chromatography of amino acids and amines the second solvent of Krebs & Hems was used (*iso*butyric acid–ammonia soln.–water) or phenol saturated with water at 18°. After a descending flow for 17–20 hr. at 22° a ninhydrin spray was used to detect amino acids and amines.

Ammonia was determined in trichloroacetic acid filtrates by distillation in Conway units followed by nesslerization. Inorganic phosphate was determined by the method of Berenblum & Chain (1938), as modified by Bartley (1953).

RESULTS

Bacterial decarboxylases

Permeability of cells. Preliminary experiments with whole cells suggested that access of L-arginine and nucleotides to the enzymes is a factor controlling reaction rates. The rate of decarboxylation of arginine by *Esch. coli* 'F' at pH 6·8 was examined under the following conditions: (*a*) whole cells which had been stored at 0°; (*b*) whole cells stored at −15°; (*c*) cell-free extracts prepared in the Mickle disintegrator (Table 1). In the absence of added nucleotide the liberation of CO_2 was very low in the whole cells; it was five times higher in the cells damaged by deep-freezing, and ten times higher in the cell-free extract. In the cells stored at

Table 1. *Effects of ATP, ADP and AMP-5′ on arginine decarboxylation in whole cells, damaged cells, and cell-free extracts of* Esch. coli '*F*'

Whole cells (7·7 mg. dry wt.) were stored at 2° or at −15°, or 4·2 mg. dry wt. of cell-free extract was stored at −15°; 0·025 M phosphate buffer, pH 6·8; 0·05 M L-arginine; 0·01 M nucleotides; final vol. 2 ml. Gas phase, N_2. Temp., 25°.

Additions	Q_{CO_2} for 0–30 min. period: Whole cells stored at 2°	Whole cells stored at −15°	Cell-free extract
—	4·6	20·7	49·0
ATP	4·4	31·2	59·0
ADP	5·5	34·2	95·2
AMP-5′	10·5	30·9	102·4

Table 2. *Effect of Cetavlon on arginine decarboxylation in whole cells and cell-free extract of* Esch. coli '*F*'

Expt. 1: 11·9 mg. dry wt. of whole cells. Expt. 2: 1·68 mg. dry wt. of cell-free extract. Phosphate buffer (0·025 M, pH 6·8); 0·025 M L-arginine; final vol. 2 ml. Gas phase, N_2. Temp., 25°.

Expt. no.	Cetavlon added (M)	Q_{CO_2} for 0–30 min. period
1	0	6·6
	5×10^{-5}	7·7
	5×10^{-4}	30·2
	5×10^{-3}	30·6
2	0	40·5
	5×10^{-4}	38·1

2°, 0·01 M AMP-5′ caused a doubling in the rate of decarboxylation; 0·01 M ADP caused an increase of about 20 % and 0·01 M ATP was ineffective. All three nucleotides gave approximately equal increases in rate (of about 50 %) with cells stored at −15°. In cell-free extracts, AMP-5′ was again the most effective stimulant, giving 105 % increase, whereas ADP and ATP increased the rate by 86 and 22 % respectively.

Cetavlon (cetyltrimethylammonium bromide), which accelerates the decarboxylation of glutamic acid in whole cells and extracts of *Cl. welchii* (Krebs, 1948; Hughes, 1949, 1950), greatly stimulated the decarboxylation of arginine in whole cells of *Esch. coli* but not in cell-free extracts (Table 2). This suggests that the effect of Cetavlon is due to an alteration of cell permeability. Most of the subsequent experiments were made on whole cells stored at 0°, to which Cetavlon (5×10^{-4} M) had been added just before the start of the incubation.

Whole cells stored at 0° showed little decline in decarboxylating activity over periods up to 4 weeks, whereas repeated deep-freezing and thawing of cells or cell-free extracts sometimes caused considerable losses in activity. These losses were rather inconsistent, varying with the organism used and the enzyme being studied.

Effects of phosphate, arsenate and adenosine 5′-phosphate. The seven strains of *Esch. coli* and *Cl. welchii* S.R. 12 were examined for the effects of 0·01 M-phosphate, arsenate and AMP-5′ on various amino acid decarboxylase activities at pH 6·4–6·8. As shown in Table 3 the decarboxylation of arginine, ornithine and lysine by the various strains of *Esch. coli* was accelerated in all cases by AMP-5′. The increases were particularly large in the cases of arginine decarboxylation by strains 'F', '4' and '8114' (+154, +193 and +149 % respectively). The largest increase caused by AMP-5′ was found during the decarboxylation of lysine by strain '4' (+255 %). In the decarboxylation of glutamate by *Cl. welchii*, there was no major effect during the first 30 min., but AMP-5′ caused a definite increase in rate in the 30–60 and 60–90 min. periods (+85 and +188 % respectively).

Appreciable increases in rate were caused by inorganic phosphate and arsenate but there were considerable strain differences. Major increases occurred in six of the fourteen combinations of organism plus amino acid examined, although small increases were sometimes obtained in the others during later periods of incubation, e.g. 30–90 min. Phosphate and arsenate usually caused increases in rate of about the same order, but only in the system of strain '4' plus ornithine did this rate equal that with AMP-5′. The decarboxylation of histidine by strain 'F' was not accelerated by any of the three substances tested.

Controls showed that in the absence of added amino acid, no CO_2 liberation was caused by incubation of organisms with phosphate, arsenate or AMP-5′.

Effect of pH. Most bacterial amino acid decarboxylases show the highest activity under slightly acid conditions, namely, pH 4–5 (Gale, 1946).

Table 4 shows that the addition of 0·01 M phosphate, or arsenate, or AMP-5′, was without effect at the optimum pH (4·5–5·15). Marked increases in the rate of decarboxylation occurred at pH 6·0 and the maximum increase was found at pH about 7·0. When a cell-free extract was used under similar conditions, AMP-5′ also caused a small increase in activity (about 15 %), on the acid side (pH 4·22) of the pH optimum (5·10 in this experiment). With extracts, the largest increases in rate also occurred at pH 6·5–7·0.

Similar results were obtained with the six other strains of *Esch. coli* tested, and with the system *Cl. welchii*–glutamate–AMP-5′. An exception was the decarboxylation of ornithine by *Esch. coli* '8571', where the accelerating effect of AMP-5′ was greater at pH 4·48 than at pH 6·70 (+158 % compared with +21 %).

Effect of the concentrations of phosphate, arsenate and AMP-5′. At pH 6·7–6·8 the rate of decarboxylation of arginine by *Esch. coli* 'F' increased with increasing concentrations of phosphate or arsenate (Table 5) and AMP-5′ (Table 6) within the ranges tested. That these stimulations were not unspecific salt effects is shown by the facts that 0·25 M-NaCl inhibited the decarboxylation by about 15 % and 0·25 M-Na_2SO_4 by about 52 % (see also Gale & Epps, 1944). The maximum rate obtained with 0·7 M-phosphate at pH 6·8 was still lower (50 %) than that observed without phosphate at the pH optimum in acetate buffer (pH 4·5).

The time curve of the decarboxylation of amino acids is known to show in most cases a progressive decline in the rate (see, for example, Fig. 1) which cannot be explained by inhibition of the reaction by its products or by the fall of the substrate concentration. Gale (1940) showed that some decline was due to the thermolability of the enzyme. The activity never disappears completely, so that the reaction, given time, goes to completion.

The accelerating effects of phosphate, arsenate and AMP-5′ are usually most pronounced during the first 30 min. of incubation, before the enzyme

Table 3. *Effects of phosphate, arsenate and AMP-5′ on bacterial decarboxylation of* L-*amino acids*

Whole cells (2–10 mg. dry wt.); 5×10^{-4}M Cetavlon; 0·025M citrate buffers, pH 6·62–6·80; 0·025M L-amino acids; 0·01M other additions at pH 6·7–6·8; final vol. 2 ml. Gas phase, N_2. Temp., 25°. Results are expressed as Q_{CO_2} for the first 30 min. incubation after addition of amino acid from the side arm.

Organism	Amino acid	Q_{CO_2} No addition	Phosphate	Arsenate	AMP-5′
Esch. coli 'F'	Arginine	42·1	76·4	74·6	107·0
	Ornithine	36·8	76·5	56·4	82·4
	Lysine	9·6	7·8	8·2	23·9
	Histidine	55·0	45·7	48·4	51·2
Esch. coli N.C.T.C. 86	Arginine	27·5	38·0	33·5	53·5
	Ornithine	16·5	25·0	19·5	31·5
Esch. coli '4'	Arginine	12·8	9·5	10·0	37·5
	Ornithine	52·0	101·5	88·0	95·0
	Lysine	27·3	42·9	55·2	97·0
Esch. coli N.C.I.B. 8571	Arginine	1·8	1·9	2·2	5·5
Esch. coli N.C.I.B. 8114	Arginine	18·8	17·8	19·6	46·8
Esch. coli 'M/48'	Arginine	16·5	11·4	12·5	36·0
Esch. coli 'T'	Arginine	7·5	7·1	8·4	13·5
Cl. welchii S.R. 12	Glutamate*	85·4	62·0	60·7	91·4

* This expt. at pH 6·42.

Table 4. *Effect of* pH *on the stimulation of the decarboxylation of arginine caused by phosphate, arsenate and AMP-5′*

Different whole-cell suspensions of *Esch. coli* 'F' were used in the three experiments: 3·7, 9·25 and 3·3 mg. dry wt. respectively in Expts. 1–3; 5×10^{-4}M Cetavlon; 0·025M L-arginine; test substances all 0·01M; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°.

Expt. no.	Buffer (M)	Final pH	Test substance	Total CO_2 liberated in 30 min. (μl.) No addition	With test substances	Increase (%)
1	Acetate–veronal (0·096)	4·75	Phosphate	1090	1162	+6·6
		5·15	Phosphate	1154	1113	0
		5·40	Phosphate	834	877	+5·2
		5·95	Phosphate	245	361	+47·4
		6·50	Phosphate	72	156	+116·8
		6·80	Phosphate	56	100	+78·6
		7·25	Phosphate	26	48	+84·6
2	Phthalate (0·05)	4·48	Arsenate	1164	1240	+6·5
	Phthalate (0·05)	5·50	Arsenate	610	695	+13·9
	Citrate (0·05)	6·62	Arsenate	117	244	+108·5
	Acetate–veronal (0·06)	7·05	Arsenate	32	62	+93·6
3	Phthalate (0·05)	4·48	AMP-5′	1087	1025	0
		4·95	AMP-5′	1034	992	0
		5·50	AMP-5′	721	765	+6·1
		5·82	AMP-5′	546	679	+24·4
	Citrate (0·05)	6·75	AMP-5′	264	423	+60·2
	Tris (0·05)	7·00	AMP-5′	138	289	+109·3
		7·40	AMP-5′	88	150	+70·5
		7·85	AMP-5′	55	64·5	+17·3

Table 5. *Effect of phosphate and arsenate concentration on the decarboxylation of* L-*arginine by* Esch. coli '*F*' *at* pH 6·7–6·8

A volume (0·25 ml.) of *Esch. coli* (7 mg. dry wt.) was stored for 7 days at −15°; 0·005 M citrate buffer, pH 6·7; 0·05 M L-arginine; sodium phosphate buffer, pH 6·8; sodium arsenate buffer, pH 6·7; final vol. 2·0 ml. Gas phase, N_2. Temp., 40°.

Phosphate (M)	Arsenate (M)	μl. of CO_2 liberated in 10 min.	30 min.	60 min.
0	0	142	195	224
0·01	0	198	306	350
0·05	0	305	555	653
0·25	0	410	931	1182
0	0·01	193	284	325
0	0·05	333	585	680
0	0·25	391	858	1072
0·01	0·01	208	360	421
0·05	0·05	362	732	888

Table 6. *Effect of AMP-5′ concentration on the decarboxylation of* L-*arginine by dialysed cell-free extracts of* Esch. coli '8114' *at* pH 6·7

Enzyme 8·4 mg. dry wt.; 0·025 M-citrate buffer, pH 6·70; 0·025 M L-arginine; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°.

AMP-5′ concn. (M)	Total CO_2 (μl.) liberated in 30 min.	60 min.	Increase in 60 min. (%)
0	44·5	62·5	—
0·0005	55·5	70·5	12·8
0·001	71·5	92	47·2
0·0025	86·5	109	74·5
0·005	107·5	127·5	104·0
0·01	147	171	173·8
0·02	250	299	378
0·04	349	462·5	640

activity has declined appreciably. After about 90 min. the rate in the presence of the accelerator often fell to that of the control vessel. Fig. 1 shows a representative rate curve where 0·005 M AMP-5′ was the accelerator.

The amounts of extra CO_2 evolved before the rates became equal were proportional to the amounts of phosphate, arsenate or AMP-5′ added. This ratio of moles of extra CO_2/mole of activator was approximately constant in any one experiment when the quantity of addition was varied. In the experiments recorded in Tables 7 and 8, ratios of 0·5:1 were found for arsenate and phosphate, and 1:1 for AMP-5′. The ratio was not altered by varying the enzyme concentration (Table 8). The same ratios were obtained with cell-free extracts, acetone-dried powder preparations and whole cells treated with Cetavlon, but not in every case. Occasionally lower ratios were obtained (i.e. less 'extra CO_2' was evolved/mole of accelerator added), particularly

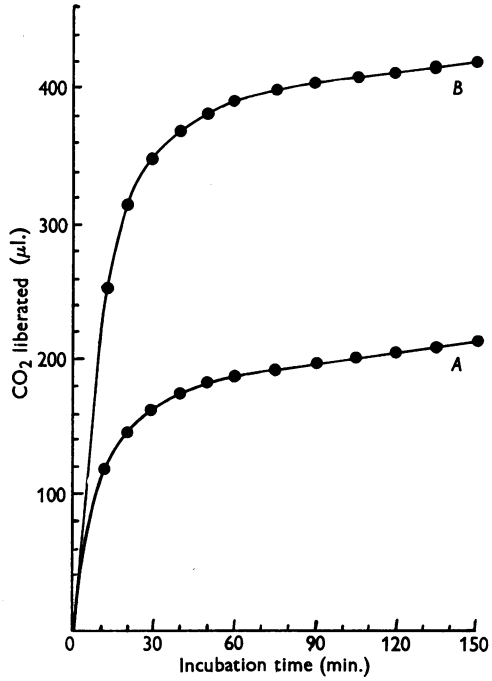

Fig. 1. Typical rate curves showing arginine decarboxylation with and without accelerator. *Esch. coli* '8114' acetone-dried powder (20 mg. dry wt.); 0·025 M-citrate buffer, pH 6·62; 0·025 M L-arginine; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°. Curve *A*, No additions; curve *B*, 0·005 M AMP-5′ added (= 224 μl.).

when fresh whole cells were used. After several days' storage, when the enzyme activity had fallen, ratios nearer to the above values were found. In other words, the accelerating effects were greater in acetone-dried powder preparations or in aged cells which showed rather feeble decarboxylase activity in the control vessels.

Paper chromatography of trichloroacetic acid extracts of the flask contents after incubation showed that AMP-5′ remained unchanged. No formation of adenosine, adenine, ribose 5′-phosphate, inorganic phosphate or ammonia was detectable, and when arginine was the amino acid decarboxylated only agmatine could be detected as end product apart from CO_2. No citrulline, ornithine or ammonia was formed.

Comparison of the effects of pyridoxal phosphate and AMP-5′. From the work of Baddiley & Gale (1945) and Umbreit & Gunsalus (1945), it is known that pyridoxal phosphate is the coenzyme of many bacterial amino acid decarboxylases, and it was of interest therefore to compare its effects with those of AMP-5′. Very small quantities of pyridoxal phosphate (e.g. 10 μg.) were sufficient to give considerable stimulation of decarboxylations; AMP-5′,

on the other hand, was required in much higher concentrations (e.g. 7 mg.) to give a similar increase (Table 9). The outstanding difference between the effects of the two substances was the pH dependence. Pyridoxal phosphate stimulated at the optimum pH of arginine decarboxylase (about pH 5), whereas AMP-5′ did not. At pH 6·7 and 7·0, on the other hand, the pyridoxal phosphate was much less effective than AMP-5′ (see Table 9). Similar observations were made with dialysed cell-free extracts of strain '8114' and with glutamic decarboxylase of *Cl. welchii* (whole cells). When both pyridoxal phosphate and AMP-5′ were added at this pH range the effects were slightly greater than the sum of the effects of the substances added alone.

Table 7. *Ratio of amounts of activator added and extra* CO_2 *liberated during arginine decarboxylation by* Esch. coli '*F*'

Whole cells of *Esch. coli* 'F' were stored for 19 days at 0° (3·7 mg. dry wt./vessel); 5×10^{-4} M Cetavlon; 0·05 M citrate buffer, pH 6·77; 0·03 M L-arginine; 0·005 M and 0·01 M phosphate, arsenate, and AMP-5′ at pH 6·7–6·8; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°. At the end of 100 min., when the rate of CO_2 evolution was the same in all vessels, 0·5 ml. of 10% (v/v) H_2SO_4 was tipped in to release bound CO_2.

	None	Phosphate		Arsenate		AMP-5′	
Additions (μmoles)	0	10	20	10	20	10	20
Total CO_2 (μmoles)	9·0	14·5	18·7	14·5	19·1	18·8	28·0
Extra CO_2 (μmoles)	—	5·5	9·7	5·5	10·1	9·8	19·0
Ratio: $\frac{\mu\text{moles extra } CO_2}{\mu\text{mole additions}}$		0·55	0·49	0·55	0·51	0·98	0·95
		Av. 0·52		Av. 0·53		Av. 0·965	

Table 8. *Effect of enzyme concentration on the ratio of AMP-5′ added to extra* CO_2 *liberated*

Acetone–ether-dried powder of *Esch. coli* '8114'; 0·025 M citrate buffer, pH 6·62; 0·025 M L-arginine; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°. Bound CO_2 was released by tipping acid when CO_2 evolution had declined to equal rates in each pair.

Enzyme added (mg.)	5		10		20	
AMP-5′ added (μmoles)	0	10	0	10	0	10
Incubation time before acid tip (min.)	150	150	105	105	90	90
Total CO_2 (μmoles)	5·2	14·9	6·5	15·3	8·8	18·1
Extra CO_2 (μmoles)		+9·7		+8·8		+9·3
Ratio: $\frac{\mu\text{moles extra } CO_2}{\mu\text{mole AMP-5′}}$		0·97		0·88		0·93

Table 9. *Comparison of the effects of AMP-5′ and pyridoxal phosphate on arginine decarboxylation by cell-free extracts and whole cells of* Esch. coli '*F*'

Buffer (0·025 M); 0·025 M L-arginine; 3 mg. dry wt. of cell-free extract in Expts. 1 and 3; 8·5 mg. dry wt. of whole cells $+5 \times 10^{-4}$ M Cetavlon in Expt. 2; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°.

Expt. no.	Buffer	pH	Additions	Q_{CO_2} for 0–30 min. period
1	Phthalate	4·95	Nil	143·2
	Phthalate	4·95	AMP-5′ (0·01 M)	142·6
	Phthalate	4·95	Pyridoxal phosphate (10 μg.)	321·0
	Phthalate	4·95	Pyridoxal phosphate (100 μg.)	350·0
	Phthalate	4·95	Pyridoxal phosphate (100 μg.) + AMP-5′ (0·01 M)	356·5
	Citrate	6·70	Nil	22·0
	Citrate	6·70	AMP-5′ (0·01 M)	54·5
	Citrate	6·70	Pyridoxal phosphate (100 μg.)	30·4
	Citrate	6·70	Pyridoxal phosphate (100 μg.) + AMP-5′ (0·01 M)	71·0
	Tris	7·00	Nil	9·0
	Tris	7·00	AMP-5′ (0·01 M)	51·3
	Tris	7·00	Pyridoxal phosphate (10 μg.)	19·3
	Tris	7·00	Pyridoxal phosphate (100 μg.)	22·0
	Tris	7·00	Pyridoxal phosphate (100 μg.) + AMP (0·01 M)	70·4

Effects of other nucleotides. During the first 30 min. of incubation of arginine decarboxylase with substrate, yeast adenylic acid increased the rate of CO_2 evolution even more than an equal concentration of AMP-5′; the final yield of extra CO_2 was usually about the same, i.e. 1 mole of yeast adenylic acid or AMP-5′ caused the liberation of 1 mole of extra CO_2. As yeast adenylic acid is known to be a mixture of adenosine 2′-phosphate and adenosine 3′-phosphate these two nucleotides were examined separately, and together. Table 10 shows that with strain '86' the relative activations in the first 30 min. of incubation were: AMP-2′ > AMP-5′ > AMP-3′; AMP-2′ was about twice as effective as AMP-3′. AMP-2′ and AMP-3′, added together in equal amounts (0·005 M), gave the same result as 0·01 M yeast adenylic acid. Paper-chromatographic analysis according to Krebs & Hems (1953) and Carter (1950) showed that none of the nucleotides had changed during the incubations and that there was no enzymic conversion of AMP-3′ into AMP-2′. Other strains of *Esch. coli* ('F', '8114' and '4') and *Cl. welchii* gave results similar to those shown in Table 10.

As shown earlier (Table 1), ATP was much less effective than ADP or AMP, except in deep-frozen cells. Further experiments showed that the effect varied with the purity of ATP samples. Impure commercial preparations of ATP containing some unidentified organic phosphates were often more effective than AMP-5′.

Inosine triphosphate and inosine 5′-phosphate, both free from other phosphates, were found to be as effective as AMP-5′. Flavin mononucleotide also caused some activation but only about 50 % of that caused by AMP-5′. Guanylic acid did not increase the rate of arginine decarboxylation by strains 'F' and '4', and was slightly inhibitory. Uridylic acid and cytidylic acid had little or no effect under these conditions.

Effects of other substances on the rate of decarboxylation. Inorganic pyrophosphate caused some acceleration in the rate of arginine decarboxylation but was not as effective as an equal concentration of inorganic phosphate. Ribose 5-phosphate was as effective as AMP-5′; glucose 1-phosphate, glucose 6-phosphate and fructose 1:6-diphosphate gave small effects. Di*iso*propyl phosphorofluoridate (0·025 M) caused less stimulation than 0·025 M inorganic phosphate during the first hour of incubation with strain 'F' but more in the second and third hours. An effect was also found with 0·025 M diethyl *p*-nitrophenyl phosphate, but this was much smaller than the inorganic phosphate effect. Tetraethyl pyrophosphate (0·025 M) gave increases similar to those of inorganic phosphate during the first hour, but the effects gradually increased so that at the end of 3 hr. the extra CO_2 obtained was more than four times that obtained with inorganic phosphate. When compared with AMP-5′, tetraethyl pyrophosphate (0·005–0·02 M) was about three times as active during arginine decarboxylation by strain 'F', and about twice as active as AMP-5′ during lysine decarboxylation by strain '4'. Even after incubation for 6 hr. with tetraethyl pyrophosphate, extra CO_2 was still being liberated, though at a reduced rate. At pH 5·2, however, 0·005 M-tetraethyl pyrophosphate completely inhibited the decarboxylation of lysine and 0·01 M inhibited the decarboxylation of arginine (Table 11).

Four organic arsenicals which caused stimulations in strain 'F' were cacodylate, phenylarsonic acid, *o*-aminophenylarsonic acid, and arsonoacetate. Their activities, compared with that given by inorganic arsenate, were 127, 74, 54 and 112 % respectively.

Substances which had no effect on the decarboxylation of arginine by strain 'F' included adenosine, adenine, inosine, glucose, carbamyl phosphate,

Table 10. *Comparative effects of the adenosine monophosphates on the decarboxylation of arginine*

Expt. 1: 10 mg. of acetone–ether-dried powder of *Esch. coli* '8114'; 0·025 M-citrate buffer, pH 6·62; 0·025 M arginine. Expts. 2 and 3: 9·45 mg. (dry wt.) of whole cells of *Esch. coli* '86'; 5×10^{-4} M Cetavlon; 0·025 M phosphate buffer, pH 6·80; 0·0125 M arginine. Final vol. in all, 2 ml. Gas phase, N_2. Temp., 25°.

Expt. no.	Additions	CO_2 liberated (μl./30 min.)	Extra CO_2 (μl.)
1	Nil	71	—
	AMP-5′ (0·005 M)	187	116
	Yeast adenylic acid (0·005 M)	221	150
2	Nil	191	—
	AMP-2′ (0·01 M)	356	165
	AMP-3′ (0·01 M)	276	85
	AMP-5′ (0·01 M)	314	123
3	Nil	233	—
	AMP-2′ (0·005 M)	333·5	100·5
	AMP-3′ (0·005 M)	282·5	49·5
	AMP-2′ (0·005 M) + AMP-3′ (0·005 M)	398	165
	Yeast adenylic acid (0·01 M)	398·5	165·5

ethylenediaminetetraacetic acid, K^+, Mg^{2+} and Mn^{2+} ions, and nucleic acid from thymus gland (British Drug Houses Ltd.). Suspensions of *Esch. coli* 'F' (20 mg. dry wt.) which had been heated for 10 min. at 100° caused some inhibition. Of other phosphates tested, dimethyl nitrophenyl phosphorothioate and dimethyl dichlorovinyl phosphate were without effect. At 0·025M concentrations octamethylpyrophosphoramide, phenyl phosphate, thiamine phosphate, thiamine pyrophosphate and α-glycerophosphate were all inhibitory. Agmatine (0·005–0·02M), one of the end products of arginine decarboxylation, had no effect on the rate of CO_2 evolution.

Plant and animal tissue decarboxylases

Three plant tissues were examined for L-glutamic acid decarboxylase activity in the filtered juice (see Methods) at pH 6·8: carrot root and the fruits of water melon and vegetable marrow. Marrow juice was about twice as active as carrot juice, and about four times as active as water-melon juice. Most of the experiments were therefore carried out with marrow juice.

When filtered marrow juice was incubated with glutamate at pH 6·8 and 25°, the addition of phosphate, arsenate or AMP-5′ had relatively small effects on the CO_2 evolution, but major effects were found with dialysed enzyme, especially with dialysed extracts of acetone-dried powder preparations. In Table 12 experiments with the latter type of enzyme preparation are shown, carried out at pH 5·50 (the optimum) and pH 6·62. At the higher pH, phosphate (0·01M) and arsenate (0·01M) each caused a threefold increase in the rate of decarboxylation, and AMP-5′ (0·01M) and yeast adenylic acid (0·01M) caused about an eightfold stimulation. At this pH, pyridoxal phosphate only doubled the rate of CO_2 evolution. On the other hand, at the optimum pH (5·5), pyridoxal phosphate was the only substance of the five compounds tested which caused a substantial stimulation. As with the bacterial enzymes, the plant enzyme was not activated by uridylic or cytidylic acids, and guanylic acid was slightly inhibitory. Marrow glutamic decarboxylase thus behaves similarly to the bacterial enzymes.

The decarboxylating activities of animal tissues are known to be much weaker than those of either bacteria or plants (see Schales, 1951). The glutamic decarboxylase of brain, and the liver enzymes decarboxylating cysteic acid and 3:4-dihydroxy-L-phenylalanine were examined at pH 6·8. Increased rates were found on addition of phosphate, arsenate, pyridoxal phosphate plus phosphate and pyrophosphate. Pyridoxal phosphate alone had almost no effect. Similar observations have already been made by other workers, e.g. Bergeret, Chatagner & Fromageot (1955); Hartman, Akawie & Clark (1955). Yeast and muscle adenylic acids caused slight inhibitions, and other substances, which stimulated the bacterial decarboxylases but were found to have no effect on the animal-tissue decarboxylases, included cacodylate, tetraethyl pyrophosphate and di*iso*propyl phosphorofluoridate.

Table 11. *Comparison of the effects of tetraethyl pyrophosphate (TEPP) and AMP-5′ on the decarboxylations of arginine and lysine at* pH 6·8 *and* 5·2

Esch. coli 'F' (11·9 mg. dry wt.) was used for arginine decarboxylation; *Esch. coli* '4' (8·3 mg. dry wt.) was used for lysine decarboxylation; 5×10^{-4}M Cetavlon; 0·0625M tris–maleate buffers; 0·05M L-arginine and L-lysine; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°.

pH	Amino acid	Incubation time (min.)	CO_2 liberated (μl.) No addition	0·01M AMP-5′	0·005M TEPP	0·01M TEPP	0·02M TEPP
6·8	Arginine	60	321	445	—	700	1047
5·2	Arginine	60	1081	1105	1276	6	—
6·8	Lysine	60	363	537	556	616	—
6·8	Lysine	360	954	1349	1377	1771	—
5·2	Lysine	60	309	327	28	14	—

Table 12. *Effects of phosphate, arsenate, adenosine monophosphates and pyridoxal phosphate on glutamate decarboxylation by dialysed extracts of marrow*

Acetone-dried powder of marrow (4·8 mg.) dialysed for 30 hr. at 0°; 0·025M-buffers; 0·025M L-glutamate; 0·01M additions except pyridoxal phosphate (20 μg.); final vol. 2·0 ml. Gas phase, N_2. Temp., 25°. Data refer to Q_{CO_2} for 0–30 min.

Buffer	Additions None	Phosphate	Arsenate	AMP-5′	Yeast adenylic acid	Pyridoxal phosphate
Citrate, pH 6·62	5·2	16·7	16·7	39·0	42·5	12·5
Phthalate, pH 5·50	28·2	32·7	30·2	28·6	28·2	43·4

Thus amino acid decarboxylases of animal tissues differ in some respects from those of bacteria and plants.

DISCUSSION

The experiments show that the activity of various amino acid decarboxylases of bacteria and plants can be increased by several inorganic and organic phosphates and arsenates. AMP-5′, inosine 5′-phosphate, AMP-2′ and tetraethyl pyrophosphate are particularly active. Activation takes place only near neutrality (around pH 6·8), and not at the pH optimum of the enzymes (around pH 4·5). In contrast, pyridoxal phosphate, the natural coenzyme, shows maximum stimulation at the optimum pH. The amino acid decarboxylases of animal tissue which have pH optima near neutrality are activated by pyridoxal phosphate but not by the organic phosphates and arsenates which are effective in bacteria and plants.

The quantities of the various compounds required for stimulation are of the order of mg./ml., whereas pyridoxal phosphate at the pH optimum acts in amounts of μg./ml. Although adenosine phosphates and inosine 5′-phosphate, as well as ribose 5-phosphate, all activate the bacterial decarboxylases, guanosine phosphate and pyrimidine nucleotides are inactive (only the mixed 2′- and 3′-phosphates were tested; the 5′-phosphates of these nucleosides were not available).

The quantitative nature of the activation of amino acid decarboxylations by AMP-5′ is comparable with other enzymes recently found to be activated by this nucleotide. For example, Tager & Rautanen (1955) found that the degree of stimulation by AMP-5′ of sulphite oxidation in oat mitochondria was a function of the AMP-5′ concentration. Madsen & Cori (1955) found that muscle phosphorylase *a* binds 4 moles of AMP-5′/mole of protein and phosphorylase *b* binds 2 moles of AMP-5′/mole of protein, and that whereas the *b* enzyme has an absolute requirement for AMP-5′, the *a* enzyme is still 65 % active in its absence.

Other enzymes requiring AMP-5′ for activity include the diphosphopyridine nucleotide-activated *iso*citric dehydrogenase of yeast (Kornberg, 1955) and of *Aspergillus niger* (Ramakrishnan & Martin, 1955). The fact that aged enzyme preparations of *Esch. coli* were more rapidly stimulated by AMP-5′ than were fresh enzymes has been reported in the present paper, and it is interesting to note that Sutherland (1955) has found that AMP-5′ and inosine 5′-phosphate increase the activity of liver polysaccharide phosphorylases to a greater extent in aged preparations than in fresh ones.

The stimulation of bacterial arginine and lysine decarboxylases by the cholinesterase inhibitor tetraethyl pyrophosphate is rather surprising, particularly as the degree of stimulation is much higher than those obtained with inorganic orthophosphate or pyrophosphate or AMP-5′. The fact that concentrations of TEPP which cause large activations at around neutral pH also inhibit the decarboxylations completely at optimum pH is of considerable interest and is being investigated further.

The results presented in this paper lead to the suggestion that there is probably a phosphate-containing substance which acts as a metabolic regulator, in that it can control the activity of the amino acid decarboxylases in the living cell. This metabolic regulator is more likely to be an organic rather than an inorganic compound, but its exact nature is open to speculation.

SUMMARY

1. The addition of cetyltrimethylammonium bromide to whole cells of *Escherichia coli* greatly increased the rate of amino acid decarboxylation. The detergent had no effect on extracts. The effect in whole cells is taken to be due to increased permeability.

2. Inorganic phosphate, arsenate and organic phosphates, especially adenosine phosphates, have been found to increase the rate of several bacterial amino acid decarboxylases when the pH is near the neutral point, but not at the optimum pH of the enzymes (pH 4–5).

3. The increases in decarboxylation rate were found to be proportional, within the range tested, to the amounts of phosphate, arsenate, or adenosine 5′-phosphate (AMP-5′) added. Two moles of phosphate or arsenate, or 1 mole of AMP-5′, caused the decarboxylation of up to 1 extra mole of amino acid. The maximum increases were obtained with aged enzyme preparations.

4. In contrast to the increases in rate obtained with AMP-5′, pyridoxal phosphate stimulated only at the optimum pH of the bacterial enzymes, and had little or no effect near the neutral point.

5. The relative activating powers of the adenosine monophosphates during the early stages of arginine decarboxylations were: adenosine 2′-phosphate > AMP-5′ > adenosine 3′-phosphate, adenosine 2′-phosphate being about twice as effective as adenosine 3′-phosphate. The total CO_2 liberated, however, was about the same. All three nucleotides were found to remain unchanged.

6. Adenosine diphosphate, inosine 5′-mono- and tri-phosphates, flavin mononucleotide, ribose 5-phosphate, and some other organic phosphates, especially tetraethyl pyrophosphate, all increased the rate of arginine decarboxylation. Similar effects were obtained with four organic arsenicals: cacodylate, phenylarsonic, *o*-aminophenylarsonic and

arsonoacetic acids. Guanylic, uridylic and cytidylic acids were among many compounds found to have little or no effect.

7. Plant tissue L-glutamic acid decarboxylases (*Cucurbita, Daucus carota*) were found to behave like the bacterial enzymes with regard to their stimulation by phosphate, arsenate, nucleotides and pyridoxal phosphate.

8. Under similar conditions the activity of animal tissue decarboxylases (L-glutamic, cysteine, 3:4-dihydroxy-L-phenylalanine) were not increased by adenine nucleotides, other phosphates or arsenates.

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A Crystallographic Study of some Derivatives of Gramicidin S

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We first began the investigation of gramicidin S in 1944 at the suggestion of Dr R. L. M. Synge, to whom we are indebted for all the material used in the research. It appeared to us to be particularly interesting as a naturally occurring peptide of relatively low molecular weight, the study of which might throw light on the arrangement of amino acid residues in proteins. Further, it crystallized with surprising ease in crystals large enough for X-ray single-crystal analysis and it contains a functional group, the δ-amino group of ornithine, which made it possible to prepare a number of derivatives of the peptide, some containing heavy atoms. The present paper gives a survey of X-ray measurements on several of these gramicidin S derivatives, undertaken as a preliminary to an attempt at a detailed X-ray analysis.

Gramicidin S was originally isolated in the form of a hydrochloride by Gause & Brazhnikova (1944). It has been shown by Synge and others (Consden, Gordon, Martin & Synge, 1947; Synge, 1948) to be a cyclic peptide built up from five different amino acid residues occurring in unimolecular proportions. These residues are L-valine, L-ornithine, L-leucine, D-phenylalanine and L-proline, written in the sequence in which they are combined in the molecule as indicated by the examination of partial hydrolysates.