Effect of Amino Acids on an Anaerobic Phosphate-Exchange Reaction in *Escherichia coli*

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(Received 16 September 1957)

Certain nucleotides, particularly adenosine mono- and di-phosphates, can stimulate amino acid decarboxylases of *Escherichia coli* at neutral pH (Eggleston, 1957). It has been investigated whether these nucleotides take part in any reactions when they increase the activity of the decarboxylases. Among the reactions tested was the incorporation of inorganic phosphate into adenosine triphosphate during amino acid decarboxylation. Experiments are described in this paper which show that a number of amino acids, particularly those undergoing decarboxylation, can increase the rate of phosphate incorporation into adenosine triphosphate.

MATERIALS

2-Amino-2-hydroxymethylpropane-1:3-diol (tris)-maleate buffers were made according to Gomori (1955); the pH was measured with a glass electrode after dilution to concentrations used in experiments. Pyridoxal phosphate was supplied by Merck and Co. Inc., Rahway, N.J., U.S.A. An HCl-hydrolysate of casein was prepared according to Barton-Wright (1952); the HCl was removed by evaporation *in vacuo*.

Nucleotides. The purity of these was examined by the paper chromatographic method of Krebs & Hems (1953); analyses are given as percentages. Adenosine 5'-phosphate (AMP) was obtained from Zellstoff-fabrik, Waldhof, Wiesbaden, Germany, and was found to be free of other phosphate-containing compounds. Adenosine diphosphate (ADP; barium salt), supplied by Schwarz Laboratories Inc., New York, N.Y., U.S.A., was also found to be chromatographically homogeneous. Three preparations of adenosine triphosphate (ATP) were used: a sodium salt from Schwarz Laboratories [ATP (Schwarz)] contained 86% of phosphorus as ATP, 8% as ADP and the rest as inorganic phosphate; a sodium salt preparation from Zellstoff-fabrik Waldhof [ATP (Waldhof)] contained 43.5% of ATP, 30.7% of ADP, 7.2% of AMP, 6% of inorganic phosphorus and 12.6% of unknown organic phosphates; a sodium salt preparation [ATP (Hems)] made by Mr R. Hems of this Laboratory as previously described (Eggleston, 1957), contained at least 98% of ATP and a trace only of ADP.

Radioactive phosphate. Carrier-free $\text{KH}_2^{32}\text{PO}_4$ in dilute HCl at about pH 2 was obtained from the Radiochemical Centre, Amersham, Bucks. It was diluted with water before use (1 mc/10 ml.) and adjusted to approx. pH 7.

Micro-organisms. *Escherichia coli* strains F, 4, 8144 and 8571 were as used previously (Eggleston, 1957).

METHODS

Suspensions of *E. coli* were grown and washed as previously described (Eggleston, 1957) and were stored at 0° as suspensions in water. Cell-free extracts were prepared in the Hughes press (Hughes, 1951) at -25° to -15°.

Incubations were carried out in Warburg vessels, the 'standard conditions' being as follows: the main compartment contained 0.0625 M-tris-maleate buffer, pH 6.8, mm-cetyltrimethylammonium bromide (Cetavlon) except in experiments with cell-free extracts, 10 μC of ^{32}P , 0.0025 M- MgCl_2 , 0.008 M-KCl, 0.005 M-sodium phosphate, pH 6.8, 0.025 M-L-amino acid (or 0.05 M-DL-amino acid) and 0.01 M-ADP or 0.0075 M-ATP (Waldhof) or AMP and ATP (Schwarz or Hems), each 0.005 M. The side bulb contained the bacteria (about 15 mg. dry wt.), which were added to the main compartment after incubation for 15 min. at 25°. The total volume in the vessels was 2 ml. The gas space was filled with N_2 and a freshly scraped stick of yellow P was placed in the centre well.

For the measurement of total CO_2 at pH 6.8 a correction was made for bound CO_2 according to Johnson (1951).

At the end of the incubation period (usually 90 min.) the cups were transferred to ice, and 0.5 ml. of 30% (w/v) trichloroacetic acid was added. After centrifuging, the clear supernatants were stored at -15°. For the determination of specific radioactivities of inorganic phosphate and nucleotides, 25 μl . samples of trichloroacetic acid extract were spotted on to the starting line of chromatogram papers, 5 μl . at a time being dried on with a current of cold air from a hair drier. The chromatographic separations were carried out as described by Krebs & Hems (1953). The spots containing ATP, ADP and inorganic phosphate were cut out from the papers and wet-ashed, and analysed for specific radioactivities (counts/min./ μg . of P) as described previously (Eggleston, 1954). Inorganic pyrophosphate (where added) was similarly determined after incubation, its R_f on the chromatogram papers being less than those of the three nucleotides.

Calculations. The extent of the esterification of inorganic phosphate to form the labile phosphate groups of ATP and ADP was calculated as those amounts of labile phosphate which exchanged with radioactive inorganic phosphate. The formula used was that of Stewart (1953). In a 'steady-state' system (i.e. where the concentrations of inorganic phosphate and labile phosphate do not change appreciably):

Total labile phosphate (μmoles) exchanged in the whole

$$\text{incubation period} = \frac{ab}{a+b} \ln \frac{bx_0}{x(a+b) - ax_0},$$

Bioch. 1958, 68

where a and b are respectively the μ moles of inorganic phosphate and of total labile phosphate (of ATP and ADP)/cup, and x_0 and x are respectively the percentages of total radioactivity in the inorganic phosphate fraction at the start and at the end of the incubation period.

RESULTS

When washed suspensions of *E. coli* were incubated anaerobically with adenine nucleotides and radioactive inorganic orthophosphate ($[^{32}\text{P}]$ orthophosphate) but without other substrates, the adenine nucleotides slowly became radioactive. Because of the limited availability of pure preparations of ADP, mixtures of pure ATP and AMP, or preparations of ATP containing some ADP (e.g. the Waldhof preparation) were used in most of the following experiments. When pure ADP as $[^{32}\text{P}]$ -orthophosphate acceptor was compared with a mixture of ATP and AMP, the results were almost identical. Time curves showed that during the incubation period (up to 120 min.) the levels of ATP and ADP did not change appreciably, but their specific radioactivities gradually increased. In other words, the hydrolysis of ATP to ADP was

balanced by its re-synthesis from ADP and inorganic phosphate and by the action of adenylate kinase. A 'steady-state' system, for the nucleotide and phosphate concentrations, thus existed.

The rate of incorporation of $[^{32}\text{P}]$ orthophosphate was increased two- to three-fold after addition of a casein hydrolysate (Table 1) or some individual amino acids (Table 2). Radioactivity was found both in ATP and ADP but not in AMP. Both labile phosphate groups of ATP had similar specific radioactivities. The observations are in accordance with the assumption that ATP is the primary nucleotide containing $[^{32}\text{P}]$ orthophosphate and that the radioactive ADP is formed by adenylate kinase. In 90 min. about one-third of the labile phosphate of ATP and ADP had exchanged with inorganic phosphate when 40 mg. of casein hydrolysate was present. Of the individual amino acids (Table 2), arginine, ornithine and cysteine were the most effective ones. Fourteen other amino acids had some activity and five, including alanine, phenylalanine, tyrosine, histidine and 3,4-dihydroxy-L-phenylalanine, were inactive. Phenylalanine, tyrosine and histidine inhibited (20–35%)

Table 1. *Effect of a casein hydrolysate on the anaerobic incorporation of radioactive orthophosphate into adenine nucleotides*

E. coli F; standard conditions, as described in the text, were used except for the addition of 0.2 or 0.4 ml. of 10% (w/v) casein hydrolysate.

Cup no.	1	2	3
Casein hydrolysate added (mg.)	0	20	40
Specific radioactivities (counts/min./ μ g. of P)			
(i) Inorganic phosphate	3390	2420	2178
(ii) Total labile phosphate of ATP and ADP	289	449	760
(ii)/(i)	0.085	0.185	0.349
Radioactivity in inorganic P (% of total)	92.55	87.60	81.18
Inorganic P/cup (μ moles)	17.00	22.35	23.62
Labile P/cup (μ moles)	16.09	17.00	15.71
Total labile P exchanged in 90 min. (μ moles)	1.365	3.280	6.035

Table 2. *Comparative effectiveness of various amino acids on incorporation of ^{32}P into adenosine triphosphate with Escherichia coli F*

Standard conditions were used, as described in the text; the blank incorporation has been deducted and the results are expressed as the percentage of the activity of L-arginine.

L-Arginine	100	L-Lysine	15
L-Ornithine	73	DL-Serine	11
L-Cysteine	73	DL-Methionine	7
L-Valine	52	L-Hydroxyproline	1
DL-Leucine	51	L-Alanine	0
Glycine	46	DL-Phenylalanine	0
DL-Tryptophan	45	3,4-Dihydroxy-L-phenylalanine	0
DL-Isoleucine	41	L-Tyrosine	0
L-Asparagine	37	L-Histidine	0
L-Aspartate	29		
L-Glutamine	28	Casein hydrolysate (10 mg.)	43
L-Glutamate	23	Casein hydrolysate (20 mg.)	126
L-Proline	22	Casein hydrolysate (40 mg.)	306
DL-Threonine	18		

Table 3. *Relative effectiveness of seven amino acids and a casein hydrolysate, compared with that of ornithine, on the ^{32}P exchange reaction with Escherichia coli 4*

Standard conditions were used, as described in the text; the blank incorporation has been deducted and the results are expressed as the percentage of the activity of L-ornithine

L-Ornithine	100
L-Lysine	64
L-Arginine	15
L-Valine	10
L-Cysteine	10
L-Glutamate	8
Glycine	3
L-Histidine	0
Casein hydrolysate (20 mg.)	12

Table 4. *Relationship between the amounts of radio-active phosphate exchanged and carbon dioxide liberated*

Standard conditions were used, as described in the text; blanks have been deducted and the results are expressed as μmoles of extra CO_2 liberated (ΔCO_2) or extra phosphate exchanged (ΔP).

<i>E. coli</i> strain	Amino acid added	ΔCO_2	ΔP	$\Delta\text{P}/\Delta\text{CO}_2$
F	L-Arginine	29.9	2.805	0.094
F	L-Ornithine	20.1	2.035	0.101
F	L-Lysine	2.2	0.410	0.186
4	L-Ornithine	39.3	5.79	0.147
4	L-Lysine	32.8	3.69	0.113
4	L-Arginine	7.9	0.84	0.106

the ^{32}P orthophosphate exchange. A smaller number of amino acids were tested with *E. coli* strain 4 (Table 3); of these ornithine and lysine were the most active, histidine again inhibited the ^{32}P orthophosphate exchange by about 35 %.

It is seen from Tables 2 and 3 that the most active amino acids (arginine, ornithine and lysine) are those which are vigorously decarboxylated at this pH (6.8) by the two organisms. In Table 4 the quotients of extra μmoles of ^{32}P orthophosphate exchanged/ μmoles of extra CO_2 liberated are shown for strains F and 4 with these three amino acids. It can be seen that the $\Delta\text{phosphate}/\Delta\text{CO}_2$ values are of about the same order (between 0.09 and 0.19); in other words, the greater the decarboxylation, the greater the labile phosphate exchange. However, some amino acids (e.g. cysteine and valine) stimulated the phosphate exchange without undergoing decarboxylation.

Decarboxylations of arginine by strain 8114, and ornithine and lysine by strain 8571, were also accompanied by increases in phosphate incorporation into ATP and ADP. In these instances $\Delta\text{phosphate}/\Delta\text{CO}_2$ values ranged from 0.05 to 0.08.

Effect of pH. For more detailed studies on the phosphate-exchange reaction and decarboxylation, strain F was selected with arginine as the substrate. The optimum pH for most bacterial amino acid decarboxylases is in the region of pH 4.5–5, with greatly reduced activity at pH 6.8 except when phosphate, arsenate or nucleotides are added (Eggleson, 1957). Lowering the pH from 6.8 to 5.2 caused a threefold increase in decarboxylation of arginine, but reduced the phosphate exchange to 15 %.

Effect of arginine concentration. Table 5 shows that maximum rates of decarboxylation were reached when the arginine concentration was about 30 $\mu\text{moles/ml.}$, and maximum rates of phosphate exchange were reached at about 15 μmoles of arginine/ml. Further increases in concentration up to 75 $\mu\text{moles/ml.}$ caused some inhibitions in phosphate exchange. In the range 5–30 μmoles of arginine/ml. the $\Delta\text{phosphate}/\Delta\text{CO}_2$ values were of about the same order in this experiment, i.e. 0.05–0.08.

Effect of amines. The end products of these amino acid decarboxylations (agmatine from arginine, cadaverine from lysine, putrescine from ornithine) were tested for their effects on the ^{32}P orthophosphate exchange reaction. No effect on the rate of decarboxylations was found when they were added in concentrations equal to that of the amino acid, but the rate of phosphate exchange was inhibited by 15–37 %. These amines depressed the 'blank' phosphate exchange as well as the exchange due to the added amino acids (see Table 6).

Effect of enzyme concentration. When cell-free extracts of *E. coli* F were used, almost no extra radioactivity appeared in ATP and ADP at very low enzyme concentrations (2.3 mg. dry wt./2 ml.). At higher concentrations (4.6 and 6.9 mg. dry wt./2 ml.) the rate of phosphate exchange increased more rapidly than the enzyme concentration, whereas the CO_2 production increased rather less than the enzyme concentration (Table 7). Other experiments were carried out with whole cells (about 15 mg. dry wt./2 ml.), to which mM-Cetavlon had been added to increase the permeability of the cells to amino acids and nucleotides (see Eggleson, 1957). When increasing the cell concentration to 40 mg. dry wt./2 ml., the Cetavlon concentration also had to be raised (to 2 mM) in order to obtain maximum rates of decarboxylation. Under these conditions both CO_2 liberation and phosphate exchange increased with the cell concentration, but the $\Delta\text{phosphate}/\Delta\text{CO}_2$ values did not change appreciably. Slightly higher concentrations of Cetavlon (3 mM) inhibited phosphate exchange completely without affecting decarboxylation. If the Cetavlon concentration was kept constant at mM and the cell concentration raised,

from 15 to 40 mg. dry wt./2 ml., CO_2 production fell and phosphate exchange was reduced in the 'blank' by 70% and not increased by arginine. This suggests that at the high cell concentrations the amount of Cetavlon was insufficient to give optimum effects and that the bacterial cells require a certain minimum concentration of Cetavlon to give maximum permeability to nucleotides, [^{32}P]-orthophosphate and amino acids.

Time curve. Time curves showed that phosphate exchange and amino acid decarboxylation do not run parallel. The decarboxylation rate declined rapidly at pH 6.8 (see also Eggleston, 1957), whereas the phosphate-exchange rate remained fairly steady over a period of 2 hr. Consequently, the $\Delta\text{phosphate}/\Delta\text{CO}_2$ ratios rose with time (Table 8). This confirms that decarboxylation is not necessary for activating the phosphate-

Table 5. *Effects of arginine concentration on the ^{32}P exchange reaction*

E. coli F; standard conditions as described in the text were used, except that in Expt. 2 incubation was for only 60 min. In cups 2 and 3 of Expt. 1 the evolution of CO_2 ceased after 30 min.

Cup no.	Expt. 1						Expt. 2			
	1	2	3	4	5	6	1	2	3	4
Arginine added (μmoles)	0	10	20	30	40	60	0	50	100	150
CO_2 evolved (μmoles)	0	10.2	20.5	28.2	36.0	44.1	0	40.3	45.7	46.2
Total labile P exchanged (μmoles)	2.96	3.53	4.47	5.20	4.96	5.07	2.47	4.20	3.80	3.36
ΔP		+0.57	+1.51	+2.24	+2.00	+2.11		+1.73	+1.33	+0.89
$\Delta\text{P}/\text{CO}_2$		0.056	0.074	0.080	0.056	0.048		0.043	0.029	0.019

Table 6. *Effect of amines on the ^{32}P exchange reaction*

Standard conditions were used, as described in the text; the amines (agmatine, putrescine and cadaverine) were added in the same concentrations as the amino acids (0.025 M).

<i>E. coli</i> strain	Additions	P exchanged (μmoles)	Inhibition (%)
F	None	4.54	—
F	Agmatine	3.87	15
F	L-Arginine	6.73	—
8571	None	1.89	—
8571	L-Lysine	3.43	—
8571	L-Lysine + cadaverine	2.15	37
8571	None	2.32	—
8571	L-Ornithine	5.06	—
8571	L-Ornithine + putrescine	3.50	31

Table 7. *Effect of enzyme concentration on ^{32}P exchange and arginine decarboxylation in cell-free extracts*

E. coli F cell-free extract was used; standard conditions were used, as described in the text.

Cup no.	1	2	3	4	5	6
Dry wt. of enzyme added (mg.)	2.3	2.3	4.6	4.6	6.9	6.9
Amino acid added	None	Arginine	None	Arginine	None	Arginine
CO ₂ liberated (μmoles)	0	10.9	0	17.9	0.7	24.0
Total labile P exchanged (μmoles)	0.425	0.440	1.34	1.65	3.24	4.68
ΔP		+0.015		+0.31		+1.44
ΔP/ΔCO ₂		0.0014		0.0173		0.062

Table 8. *Effect of the period of incubation on ^{32}P exchanged and the decarboxylation of arginine and lysine*

E. coli strain F with arginine, strain 4 with lysine; standard conditions were used, as described in the text; the blank CO_2 evolution and ^{32}P exchange have been deducted.

Amino acid	Incubation period (min.)	CO_2 liberated (μmoles)	P exchanged (μmoles)	P/ CO_2
L-Arginine	0-60	25.6	1.227	0.048
L-Arginine	60-90	5.0	0.723	0.145
L-Arginine	90-120	3.3	0.580	0.176
L-Lysine	0-60	32.2	1.168	0.036
L-Lysine	60-90	10.4	0.792	0.076
L-Lysine	90-120	4.6	0.705	0.153

exchange reaction. The fall in decarboxylase activity at pH 6.8 is not due to inhibition by end products and has not been satisfactorily explained.

Effect of phosphate concentration and pyridoxal phosphate. When the concentration of inorganic phosphate was doubled there was a small increase in CO_2 production and labile phosphate exchanged during arginine decarboxylation by strain F. The $\Delta\text{phosphate}/\Delta\text{CO}_2$ value remained the same.

The addition of pyridoxal phosphate (20 $\mu\text{g.}$) had very little effect on the $\Delta\text{phosphate}/\Delta\text{CO}_2$ values at pH 6.8 or 5.2. It has previously been shown (Eggleston, 1957) that this coenzyme has its maximum effect on bacterial amino acid decarboxylases at the optimum pH (about 4.5) and that it has very little effect on CO_2 production near the neutral point.

Incorporation of radioactive orthophosphate into inorganic pyrophosphate. When [^{32}P]orthophosphate, AMP and inorganic pyrophosphate were incubated with *E. coli* F and arginine, only traces of isotope appeared in the inorganic pyrophosphate. No ATP or ADP was detectable by chromatographic methods and the concentration of inorganic pyrophosphate was not appreciably altered. Under

the same conditions incorporation of [^{32}P]orthophosphate into nucleotides took place when [^{32}P]orthophosphate, AMP and ATP were present (Table 9, Expts. 1 and 2). A separate experiment in which [^{32}P]orthophosphate, inorganic pyrophosphate, AMP and ATP were all incubated together showed a labelling of inorganic pyrophosphate almost as high as the labile phosphate of ATP (Table 9, Expt. 3). The interpretation of these results appears in the Discussion.

Effect of inhibitors. Taylor & Gale (1945) showed that the bacterial amino acid decarboxylases are inhibited by semicarbazide, hydrazine and hydroxylamine. At concentrations of semicarbazide and hydrazine (mm) at which amino acid decarboxylation was only slightly inhibited, the accompanying phosphate exchange was greatly or completely inhibited (Table 10). The 'blank' phosphate exchange (without amino acid) was not inhibited by these reagents. Hydroxylamine (mm) gave similar results, but with higher concentrations (2 mm) CO_2 production was completely inhibited, as well as phosphate exchange. Here again the 'blank' phosphate exchange was not inhibited (Table 11).

Table 9. Conditions for the incorporation of ^{32}P into inorganic pyrophosphate

E. coli F; standard conditions were used except for the additions of nucleotides [0.01 M-AMP in Expt. 1, 0.005 M each of AMP and ATP (Schwarz) in Expts. 2 and 3] and inorganic pyrophosphate (0.01 M in Expt. 1, 0.005 M in Expt. 3). Expts. 1 and 2 were run simultaneously from the same bacterial suspension. Results refer to specific radioactivities (counts/min./ $\mu\text{g.}$ of P). PP: inorganic pyrophosphate.

Additions	Expt. 1		Expt. 2		Expt. 3	
	(a)*	(b)*	(a)	(b)	(a)	(b)
	AMP PP	AMP PP	AMP ATP	AMP ATP	AMP ATP PP	AMP ATP PP
Orthophosphate	2640	2580	3390	2663	1800	1236
Pyrophosphate	22	36	0†	0	175	415
Labile P (ATP + ADP)	0	0	289	501	223	465
PP/P	0.008	0.014	0	0	0.097	0.336
Labile P/P	0	0	0.085	0.188	0.124	0.376

* (a) Without arginine; (b) with arginine.

† In this table zero signifies non-detectable quantities.

Table 10. Effect of semicarbazide and hydrazine on arginine decarboxylation and the phosphate-exchange reaction

E. coli F; standard conditions were used, as described in the text, semicarbazide and hydrazine were added at mm concentration.

Cup no.	...	...	...	...	1	2	3	4	5	6
Amino acid added	...	...	...	...	None	Arginine	None	Arginine	None	Arginine
Other additions	...	...	...	...	None	None	Semi-carbazide	Semi-carbazide	Hydrazine	Hydrazine
CO_2 liberated (μmoles)	...	...	...	...	1.6	35.0	0.9	28.5	0.6	24.2
ΔCO_2	...	...	...	...	—	+33.4	—	+27.6	—	+23.6
Total labile P exchanged (μmoles)	...	...	...	...	3.47	10.02	3.78	6.87	5.10	4.48
ΔP	...	...	...	...	—	+6.55	—	+3.09	—	-0.62
$\Delta\text{P}/\Delta\text{CO}_2$	...	...	...	...	—	0.196	—	0.112	—	0

Table 11. *Effect of hydroxylamine on decarboxylations and phosphate exchange*

E. coli strain 4 with lysine, strain F with arginine; standard conditions were used, as described in the text, except that in Expt. 2 incubation was for 120 min.

Additions	CO ₂ liberated (μmoles)	ΔCO ₂	P exchanged (μmoles)	ΔP	ΔP/ΔCO ₂
Expt. 1					
None	1.5	—	2.32	—	—
Arginine	29.8	+28.3	3.52	+1.20	0.042
Hydroxylamine (mm)	0.4	—	2.27	—	—
Hydroxylamine (mm); arginine	16.9	+16.5	2.44	+0.17	0.010
Expt. 2					
None	0	—	4.19	—	—
Lysine	47.2	+47.2	6.85	+2.66	0.056
Hydroxylamine (2 mm)	0	—	4.20	—	—
Hydroxylamine (2 mm); lysine	1.2	+1.2	3.81	-0.39	0

Table 12. *Effect of 2:4-dinitrophenol and iodoacetate on decarboxylations and phosphate exchange*

E. coli strain F with arginine, strain 4 with lysine; standard conditions were used, as described in the text. Results for Expt. 2 are the averages of duplicates.

Additions	CO ₂ liberated (μmoles)	ΔCO ₂	P exchanged (μmoles)	ΔP	ΔP/ΔCO ₂
Expt. 1					
None	1.5	—	2.32	—	—
Arginine	29.8	+28.3	3.52	+1.20	0.042
Dinitrophenol (0.1 mm)	0.8	—	2.39	—	—
Dinitrophenol (0.1 mm); arginine	29.7	+28.9	4.16	+1.77	0.061
Expt. 2					
None	0.4	—	1.43	—	—
Arginine	40.8	+40.4	3.74	+2.31	0.057
Dinitrophenol (0.1 mm); arginine	39.3	+38.9	3.86	+2.43	0.063
Iodoacetate (10 mm); arginine	41.0	+40.6	1.53	+0.10	0.002
Expt. 3					
None	0	—	6.32	—	—
Lysine	52.5	+52.5	14.71	+8.39	0.160
Iodoacetate (10 mm)	0	—	5.70	—	—
Iodoacetate (10 mm); lysine	49.0	+49.0	9.20	+3.50	0.072

Table 13. *Effect of sodium fluoride on decarboxylations and phosphate exchange*

E. coli strain 4 with lysine, strain F with arginine; standard conditions were used, as described in the text. Results for Expt. 2 are the averages of duplicates.

Additions	CO ₂ liberated (μmoles)	ΔCO ₂	P exchanged (μmoles)	ΔP	ΔP/ΔCO ₂
Expt. 1					
None	0	—	6.32	—	—
Lysine	52.5	+52.5	14.71	+8.39	0.160
Sodium fluoride (0.02 m)	0	—	6.59	—	—
Sodium fluoride (0.02 m); lysine	52.2	+52.2	16.99	+10.40	0.199
Expt. 2					
None	0.4	—	1.43	—	—
Arginine	40.8	+40.4	3.74	+2.31	0.057
Sodium fluoride (0.02 m); arginine	39.2	+38.8	4.78	+3.35	0.086

2,4-Dinitrophenol (0.1 mM), which is known to uncouple oxidative phosphorylations, had no effect on arginine decarboxylation or on the 'blank' phosphate exchange, but increased the phosphate exchange in the presence of arginine by 10–45% (Table 12). Fluoride has a similar effect (Table 13). In the presence of lysine, NaF (0.02 M) increased the Δ phosphate/ Δ CO₂ value by about 24%, and this figure (0.199) was the highest obtained in the present work. Iodoacetate (0.01 M) inhibited phosphate exchange without affecting the decarboxylations of arginine or lysine (Table 12).

DISCUSSION

An anaerobic exchange reaction leading to the incorporation of [³²P]orthophosphate into ATP has been shown to occur in animal tissues by Boyer, Falcone & Harrison (1954), and subsequently by Cohn & Drysdale (1955), Swanson (1956), Boyer, Luchsinger & Falcone (1956), Cooper & Lehninger (1957) and Plaut (1957). A similar reaction in *E. coli* has been briefly reported by De Moss & Novelli (1956). The experiments reported in the present paper show that the exchange reaction in *E. coli* can be accelerated by the addition of amino acids, both in whole cells and in extracts.

The addition of mixtures of amino acids, such as a casein hydrolysate (Tables 1 and 2), or individual amino acids (Tables 2 and 3), caused increases in the rate of phosphate exchange up to about three-fold. Of twenty-three amino acids tested, seventeen were active, the most effective being arginine, ornithine and cysteine with *E. coli* strain F, ornithine and lysine with strain 4. Arginine was also active with strain 8144, ornithine and lysine with strain 8571. Among the most effective amino acids were those which were rapidly decarboxylated, and there was some parallelism between the amounts of labile phosphate exchanged and carbon dioxide liberated (see Table 4). However, decarboxylation of an amino acid is not essential for the stimulation of the phosphate-exchange reaction. Thus cysteine, valine, leucine and glycine promoted the phosphate exchange without undergoing decarboxylation, although the effect of these amino acids was usually much weaker than that of those being rapidly decarboxylated (Tables 2 and 3). With strain F cysteine was as active as ornithine, but with strain 4 it had only one-tenth of the activity of ornithine.

There was no simple stoichiometry between the amounts of phosphate incorporated into ATP and of arginine, ornithine or lysine decarboxylated; at most, 0.2 μ mole of phosphate was exchanged/ μ mole of carbon dioxide released. Even with the same amino acid, there were large variations in the Δ phosphate/ Δ CO₂ values obtained in different

experiments (0.04–0.20). This may be accounted for by the assumption that the decarboxylases and the phosphate-exchange reaction are independent systems. This is borne out by the effect of pH, time curves (Table 8), effects of amines (Table 6) and the effects of amino acid and enzyme concentrations (Tables 5 and 7), and by the effects of inhibitors. Thus the phosphate-exchange system was more sensitive to the carbonyl-reacting substances, semicarbazide, hydrazine and hydroxylamine, than were the decarboxylases. The inhibition of phosphate exchange by iodoacetate suggests that thiol groups are necessary for the activity of this system.

The exchange reaction described in this paper is different from the rapid exchange reaction between inorganic pyrophosphate and ATP in the presence of amino acid mixtures incubated with rat liver (Hoagland, 1955) or bacterial preparations (De Moss & Novelli, 1956). The latter authors, examining the reaction in *E. coli* in detail, found only eight amino acids individually effective in increasing the rate of pyrophosphate exchange. Isoleucine, leucine, valine, tyrosine and methionine were the most effective, phenylalanine, histidine and tryptophan less active, and arginine and lysine were completely ineffective. In contrast the present data show that tyrosine, histidine and phenylalanine cause no increases in phosphate incorporation and, in fact, were slightly inhibitory (20–35%).

De Moss & Novelli also noted that inorganic orthophosphate was incorporated into ATP in the presence of amino acid mixtures, but this was only about one-thirtieth of the pyrophosphate-exchange reaction, and was not further studied by them. The rate observed in the present experiments for the orthophosphate exchange is of the same order as that reported by De Moss & Novelli.

At pH 5.2, which is nearer the optimum for the decarboxylases (4.5–5), the phosphate-exchange reaction was only 15% of that at pH 6.8; in contrast De Moss & Novelli found about 50% of maximum activity of the pyrophosphate-exchange reaction at the lower pH. Another difference between the phosphate- and pyrophosphate-exchange reactions lies in the concentrations of amino acids necessary for maximum activation. De Moss & Novelli found that 'saturation' with some amino acids was reached at low concentrations, e.g. 0.25 μ mole of leucine/ml. for the pyrophosphate-exchange reaction. Maximum rates of phosphate exchange were only reached with about 15 μ moles of arginine/ml. (see Table 5).

The experiments in Table 9 show that the incorporation of [³²P]orthophosphate into ATP does not involve a primary conversion of phosphate into pyrophosphate with subsequent incorporation via the 'Hoagland reaction'. Thus no formation of

radioactive ATP from [^{32}P]orthophosphate, AMP and inorganic pyrophosphate could be detected unless ATP (non-radioactive) was also added. The results support the assumption that under the experimental conditions, ^{32}P must first be incorporated into both labile phosphate groups of ATP before radioactivity can appear in inorganic pyrophosphate (PP), in the following manner:

- (i) adenosine pyrophosphate + [^{32}P]orthophosphate
 $\rightleftharpoons$ adenosine PP [^{32}P]orthophosphate
- (ii) adenosine PP [^{32}P]orthophosphate
 $\rightleftharpoons$ adenosine phosphate + adenosine PP
- (iii) adenosine phosphate [^{32}P]orthophosphate
 $\rightleftharpoons$ adenosine phosphate [$^{32}\text{P}_2$]pyrophosphate
- (iv) adenosine phosphate [$^{32}\text{P}_2$]pyrophosphate
 $\rightleftharpoons$ adenosine phosphate + [$^{32}\text{P}_2$]pyrophosphate.

Reactions (i) and (iii) would be the slow phosphate-exchange reactions reported for the incorporations in this paper, and reactions (ii) and (iv) respectively the rapid reactions of adenylate kinase and the 'Hoagland reaction'. As arginine, ornithine and lysine do not affect reaction (iv), its high activity would correspond to the large 'blank' exchange reaction found by De Moss & Novelli in the absence of added amino acids.

The phosphate exchange between the terminal phosphate of ATP and inorganic phosphate was taken by Boyer *et al.* (1954) to be due to a reversible step in oxidative phosphorylation. Whether the acceleration of the exchange reaction by amino acids is concerned with the systems responsible for oxidative phosphorylation is an open question. It is noteworthy that, whereas the phosphate-exchange reaction observed by Boyer *et al.* (1954) and Cooper & Lehninger (1957) was inhibited by 2:4-dinitrophenol (0.3 mM and 5 μM respectively), a similar reaction studied by Plaut (1957) on purified liver extracts was insensitive to 2:4-dinitrophenol. In the present work on *E. coli* the exchange reaction accelerated by amino acids was not inhibited by 2:4-dinitrophenol (0.1 mM) but was slightly increased (Table 11).

SUMMARY

1. Adenosine triphosphate, ^{32}P -labelled inorganic orthophosphate and individual amino acids were added to *Escherichia coli* preparations incubated anaerobically and the rate of exchange between inorganic phosphate and the labile phosphate of adenosine triphosphate was measured; the concentration of phosphate and nucleotides remained constant.

2. Arginine, ornithine and lysine, which were

rapidly decarboxylated, were found to be the most effective activators of phosphate exchange. The highest values for μmoles of phosphate exchanged/ μmole of carbon dioxide liberated were about 0.2.

3. Although some parallelism was observed between the amounts of phosphate exchanged and carbon dioxide liberated, the decarboxylation of an amino acid was not essential for an increased phosphate exchange. Amino acids which accelerated the phosphate exchange without undergoing decarboxylation included cysteine, valine, leucine, glycine and tryptophan.

4. The phosphate-exchange reaction was very much more rapid at pH 6.8 than at pH 5.2, although the lower pH is nearer to the optimum pH of the decarboxylases (about 4.5).

5. Arginine, cadaverine and putrescine caused some inhibition of the phosphate-exchange reaction.

6. The phosphate-exchange reaction was more sensitive to mm-hydrazine, -semicarbazide and -hydroxylamine than were the decarboxylases. Sodium fluoride (0.02 M) and 2:4-dinitrophenol (0.1 mM) did not inhibit, but caused small increases in the rate of phosphate exchanged. Iodoacetate (0.01 M) inhibited the phosphate exchange but not the decarboxylases.

7. The enzyme systems responsible for phosphate incorporation into adenosine triphosphate, and for the pyrophosphate exchange with adenosine triphosphate described by Hoagland (1955) and De Moss & Novelli (1956), are not identical, because different amino acids are required to activate the two systems.

8. The possibility is discussed that the phosphate-exchange reaction described here is similar to that observed in animal tissues by Boyer, Falcone & Harrison (1954).

The author wishes to thank Professor H. A. Krebs, F.R.S., for his helpful criticisms. This work was aided by a grant from the Rockefeller Foundation to this Department.

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Effect of Ischaemic Necrosis and Autolysis *in vitro* on the Soluble Proteins of Rat Kidneys

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(Received 8 August 1957)

The structural changes undergone by the cells in the course of coagulation necrosis have been investigated in detail by many authors (Arnheim, 1890; Heidenhain, 1907; Schmaus & Albrecht, 1895; Schottlander, 1888, 1891). Although many papers have been published on the biochemical changes occurring during the necrotic process, an explanation, on a biochemical basis, of the events taking place in this pathological condition is still lacking. In particular, the data so far available on the fate of the protein components of the cells in this condition are essentially analogous as they deal mainly with the autolysis *in vitro* under aseptic conditions. These investigations deal particularly with the behaviour of various enzyme systems and with the substances appearing during the hydrolytic cleavage of the proteins (see the extensive reviews of Bradley, 1922, 1938; Pozzi, 1935). The disappearance of the cellular proteins, in the course of necrosis, was thus referred to digestion of the protein components due to proteolytic enzymes still active in the dead cells (Bradley, 1922, 1938).

No investigations on the biochemical changes of the soluble proteins, during the course of necrosis, have been carried out so far by direct analytical procedures. In this paper the soluble proteins of normal kidneys and of kidneys in the course of ischaemic necrosis and autolysis *in vitro* have been studied, mainly by paper electrophoresis.

EXPERIMENTAL

Male albino rats of the Italic strain, weighing 150–180 g., bred in this Department and fed on a standard diet (Randoin & Causeret, 1947), were used.

Ligation of the renal vessels. Ligation of the left renal artery and vein was carried out, under light ether anaesthesia, with sterile silk thread. To ensure partial drainage of the blood trapped in the vessels of the organ, the renal vein was severed distally to the ligature.

Transplantation of the kidneys to the peritoneal cavity. Animals were killed by exsanguination under light ether anaesthesia and the body was perfused through the ascending aorta with about 200 ml. of sterilized ice-cold 0.9% NaCl soln. The kidneys were removed, weighed and transferred under aseptic conditions to the peritoneal cavities of other rats.

Aseptic autolysis in vitro. Organs perfused as described above were put in sterilized glass-stoppered test tubes containing some drops of chloroform. Direct contact of the antiseptic was avoided by suspending the organs on small glass rods. Autolysis was carried out in an oven at 37°.

Extraction of the soluble proteins. The weighed organs were dispersed in 1 vol. of water, adjusted to pH 7.8, with a Potter-Elvehjem glass homogenizer kept in crushed ice. The dispersion was centrifuged for 1 hr. at 25 000 g in a refrigerated angle centrifuge (Espirouette Phywe, Phywe A.-G., Göttingen) and the supernatant fluid again centrifuged for 1 hr. with the same centrifugal force. The supernatant fluid was stored in the cold room at 2° and used for the electrophoretic analysis.

Determination of the total and soluble protein contents of the tissue dispersions. Proteins from the whole homogenate and supernatant fluid were precipitated with 5% (w/v) trichloroacetic acid; digestion was performed in the presence of potassium persulphate; NH_3 was distilled in the Markham (1942) apparatus and collected in 0.0143 N- H_2SO_4 for back titration with 0.0143 N-NaOH, Tashiro's indicator (King, 1951) being used.

Paper electrophoresis. This was carried out in an horizontal-strip-type of apparatus with a boric acid- Na_2SO_4 -NaOH buffer [0.154 M, pH 8.6, I 0.12 (P. Adjutantis, personal communication).]

Strips of paper (Whatman no. 1; 12 cm. $\times$ 30 cm.) were allowed to equilibrate in the apparatus and then the fluids were applied as a narrow band near to the cathode end. Separation was carried out with a constant potential of 135–150 v and a current, at the beginning of the experiment, of about 4 mA for a single strip. The strips, dried in an oven at 110° for 30 min., were stained with Amido-black 10B according to Grassman, Hannig & Knedel (1951). The relative percentages of the separated components were