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Purine and Pyrimidine Transaminases in *Escherichia coli**

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Biological conversion of amino purines and pyrimidines into their hydroxy compounds has hitherto been ascribed exclusively to the action of hydrolytic deaminases. The report that suspensions or extracts of *Escherichia coli* (strains B and Crookes) catalyse transamination between α -oxoglutarate and adenine, guanine or cytosine (Gunsalus & Tonzetich, 1952) would point to the existence of important alternate metabolic pathways for these

constituents of nucleic acids. The transamination reaction, as usually understood, involves an intermolecular oxidation-reduction. This reaction is not possible with an intact purine or pyrimidine ring; therefore transamination of these compounds must involve a mechanism not previously described or must be preceded by a change in the ring such as reduction. It seemed therefore of importance to repeat and to extend the experiments of Gunsalus & Tonzetich (1952). We have not been able to confirm their observations by employing similar bacteria and similar experimental conditions. Nor was transamination evident, when, in a limited number of experiments, mammalian-liver homogenates or slices replaced bacterial preparations.

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MATERIALS AND METHODS

Chemicals. The chemicals used were obtained as follows: adenosine and cytidine (ribosides), cytosine and ninhydrin, from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; adenine and glutamic acid, CFP brand, from the California Foundation for Biochemical Research; reduced diphosphopyridine nucleotide (DPNH) from Sigma Chemical Co., St Louis, Mo., U.S.A.; α -oxoglutaric acid, lot IV-8-A from the California Foundation for Biochemical Research; pyridoxal phosphate (Ca salt, 100 mg. = approx. 70 mg. of pyridoxal) from the Merck Institute for Therapeutic Research.

Bacterial suspensions and extracts. A loop of growth from a slant of *E. coli* (strains B and Crookes) was transferred to fresh nutrient agar and incubated at 37° for 18–24 hr. Loops of the actively growing bacteria were used to seed 500 ml. of broth having the same composition as the subsequent medium. In 24 hr. dense growth of bacteria resulted and all of the culture was used to inoculate 10–20 l. of medium. The composition of the latter medium and the growth and harvesting of cells were as described by Gunsalus & Hand (1941). The final bacterial suspensions were freeze-dried and stored at -15°. Several dried batches of the same strain of bacteria were combined. Just before use, weighed amounts of the dried cells were suspended in water or in phosphate buffer and used without further treatment. Cell-free extracts were occasionally prepared by the method of Gunsalus & Tonzetich (1952).

From a slant of *E. coli*, American Type Culture (A.T.C.) no. 4157, cells were grown and harvested as described by Cohen (personal communication). These cells were freeze-dried and stored at -15°. Suspensions of A.T.C. no. 4157 in acetate buffer pH 5 (prepared just before use) were used as a source of glutamic acid decarboxylase.

Liver homogenates and slices. Homogenates of rabbit liver (1 vol. of liver and 4 vol. of either water or phosphate buffer) were prepared according to the method of Potter & Elvehjem (1936).

Slices (approx. 0.5 mm. thick and weighing 50–75 mg.) were cut from rat liver with a Stadie-Riggs microtome. All the operations with fresh tissue were carried out in a cold room at 5–6°. Approx. 30 min. elapsed between killing the animal and starting the reaction by adding homogenate or slices to the medium.

Conditions for measuring transamination

In all four methods which follow, the α -oxoglutarate and the amino compounds were dissolved in 0.05 M-phosphate buffer, pH 7.4. All phosphate buffers were made by mixing KH_2PO_4 and Na_2HPO_4 .

Method 1. The substrate control consisted of the following: 0.5 ml. of pyridoxal phosphate (0.06 μ mole), 1 ml. of 0.05 M-phosphate buffer, pH 7.4, 0.5 ml. of α -oxoglutarate (40–80 μ moles), and 1 ml. of amino compound (25 μ moles of adenine, cytosine, adenosine or cytidine). For the enzyme control, the amino compound was replaced by 1 ml. of enzyme preparation. For the complete system, the phosphate buffer was replaced by 1 ml. of enzyme preparation. The enzyme preparation was 20% (v/v) of rabbit-liver homogenate in 0.05 M-phosphate buffer, pH 7.4.

The reaction was carried out in 15 ml. screw-topped culture tubes which were continuously shaken at 37°. After

1 hr., 7 ml. of ethanol was added to each tube and the insoluble material centrifuged off. The supernatant fluid was evaporated to dryness. The dry residue was dissolved in 1 ml. of water. Samples were spotted on Whatman filter paper no. 1. Phenol-water (80:20, w/v) mixture was employed as the solvent for descending chromatography. The dried paper was treated with ninhydrin. The amount of glutamate formed was based on visual comparison with known amounts of glutamic acid (0.013–0.156 μ mole) simultaneously spotted and run together with the unknowns.

Method 2. This was essentially the method of Gunsalus & Tonzetich (1952). The composition of the solution for the substrate control was as follows: 0.3 ml. of pyridoxal phosphate (0.04 μ mole), 0.1 ml. of M-phosphate buffer, pH 8.3, 0.4 ml. of α -oxoglutarate (25 μ moles) plus amino compound (32 μ moles), and 0.2 ml. of water. For the enzyme control, the amino compound was omitted and the water replaced by 0.2 ml. of a bacterial extract or suspension (15 mg. dry wt. of bacteria) in 0.1 M-phosphate buffer, pH 8.3. For the complete system the water was replaced by 0.2 ml. of the bacterial extract or suspension.

Conditions for the reaction were as described for Method 1. The reaction was stopped by the addition of 0.1 ml. of melted trichloroacetic acid. After centrifuging, 50 μ l. portions of the clear supernatant were spotted on paper and all subsequent steps were carried out as in method 1. Glutamic acid standards were made up in trichloroacetic acid solution.

Method 3. The composition of the test solution was essentially the same as in method 2 except that 0.2 ml. of DPNH (5 μ moles) was added to all reaction tubes and the bacterial suspensions or extracts were made up in 0.1 M-phosphate buffer, pH 7.4. The reaction was stopped by the addition of 1 ml. of ethanol. The clear supernatant fluid produced by centrifuging was used for spotting and the subsequent steps in the development of the chromatogram were the same as in method 1.

Elution of the ninhydrin-coloured areas with an R_F corresponding to glutamate and spectrophotometric determination of glutamate were carried out essentially by the method of Giri, Radhakrishnan & Vaidyanathan (1952).

Method 4 (manometric method). The substrate control contained the following: 0.3 ml. of pyridoxal phosphate (0.12 μ mole), 1 ml. of 0.1 M-phosphate buffer, pH 8.3, 0.5 ml. of α -oxoglutarate (40 μ moles), 1 ml. of amino compound (25 μ moles). The enzyme control contained 1 ml. of bacterial preparation or liver homogenate in place of the buffer, and the amino compound was replaced by 1 ml. of 0.05 M-phosphate buffer, pH 7.4. For the complete system the pH 8.3 buffer was replaced by 1 ml. of enzyme solution. The amino compound was adenine, cytosine, adenosine or cytidine.

The bacterial enzyme preparations used were suspensions or extracts of *E. coli* (strains B or Crookes). Suspensions contained 15 mg. of dried cells; extracts were derived from 24 mg. of cells. Both were made up in 0.1 M-phosphate buffer, pH 8.3. The enzymic preparation from rabbit liver was a 20% (v/v) homogenate in 0.1 M-phosphate buffer, pH 8.3.

The reaction was carried out in Warburg flasks at 37° for 1 or 2 hr. The temperature of the flasks was then rapidly brought to 100° and maintained at this level for 10 min. to stop the reaction. The contents were cooled and then brought to pH 5 by the addition of 0.5 ml. of 3 M-acetate buffer,

pH 4.8, and glutamate was determined according to a modification of the usual manometric procedure (Gale, 1945; Krebs, 1948), whereby *E. coli* A.T.C. no. 4157 is employed (Dr P. Cohen, personal communication) instead of *Clostridium welchii* as a source of glutamic acid decarboxylase.

Recovery of formed glutamate was estimated by replacing the amino compounds in some flasks with known amounts (3–8 μ moles) of glutamate; evolution and measurement of CO_2 derived from glutamate also served to test the activity of the glutamic acid decarboxylase.

EXPERIMENTAL AND RESULTS

It was impossible to prepare a solution containing 25 μ moles of guanine/ml. at pH 8.3, as specified by Gunsalus & Tonzetich (1952). Aqueous solutions of guanine at 40° contain about 0.26 μ mole/ml. (Wood, 1903). A concentration of not more than 6 μ moles of guanine/ml. at pH 8 could be obtained by resorting to colloidal suspensions containing gelatin (Schmidt, 1932). The uncertainty of the actual guanine concentration in such colloidal solutions prompted us to omit guanine from our experiments.

Table 1 lists the results obtained in experiments similar to but not identical with those of Gunsalus & Tonzetich (1952). It may be observed that little or no formation of glutamate could be detected on chromatograms when adenine, cytosine, adenosine or cytidine, together with α -oxoglutarate, were incubated with bacterial preparations, rabbit-liver homogenates or rat-liver slices. Thus evidence for transamination involving adenine, cytosine, their respective nucleosides and α -oxoglutarate in solutions containing enzyme preparations was not obtained.

Manometric determinations of glutamate, with a preparation of *E. coli*, A.T.C. no. 4157, as a source of glutamic acid decarboxylase, confirmed the

negative results of chromatography for rabbit-liver homogenate and *E. coli* B.

The chromatographic methods for measuring glutamic acid were tested by replacing the amino compound with glutamic acid in solutions which contained enzyme preparations and in those which did not. The activity of A.T.C. no. 4157 suspensions was checked in each experiment with known amounts of glutamate.

Recoveries of added glutamate in various experiments ranged from 63 to 125 % from the chromatographic experiments and from 73 to 110 % for the manometric experiments (Table 2).

Not included in Table 2 are the results of a large series of experiments in which 25 μ moles of glutamate were added to solutions which contained suspensions or extracts of both *E. coli* B and Crookes, and to solutions which were enzyme-free. Recoveries of glutamate by chromatography (Method 3) in all of these experiments were in the range 88–116 %.

However, the distinct possibility remained that small amounts of glutamate could actually have been formed, and have remained undetected either because of analytical limitations or because the amino acid was itself metabolized by the bacteria. To test this possibility, 4 or 8 μ moles of glutamate were incubated with *E. coli* Crookes as described in method 3. It may be seen (bottom of Table 2) that whereas approx. 2.5 μ moles or 63 % of glutamate were recovered when 4 μ moles were incubated with *E. coli* Crookes the corresponding average recovery was 7.1 μ moles or 89 % when 8 μ moles were incubated. This is in contrast to recoveries of 90–98 % and 100–106 % in similar experiments in which bacterial preparations were not added to the media. The higher recoveries obtained when larger amounts of glutamate are employed may tend to obscure small but fairly constant losses. These losses in the

Table 1. *Glutamate formation from adenine, cytosine and their nucleosides*

All listed results are the average of duplicate complete runs. The last two expts. were done manometrically; the remainder were done chromatographically. For conditions see Methods.

Enzyme source	Method no.	Glutamate formed (μ moles)			
		Enzyme blank	+ Adenine	+ Cytosine	+ Adenosine + Cytidine
Rabbit-liver homogenate	1	No discernible increase of area or intensity of coloured spots over those of enzyme controls			
<i>E. coli</i> B, extract	2				
<i>E. coli</i> Crookes, extract	2				
<i>E. coli</i> B, suspension	3	Trace	Trace	Trace	—
<i>E. coli</i> B, suspension	3	1.3	1.3	1.0	0.2
<i>E. coli</i> B, extract	3	0.6	0.7	1.6	0.2
<i>E. coli</i> Crookes, suspension	3	Trace	Trace	Trace	—
<i>E. coli</i> Crookes, suspension	3	0.7	0.7	0.6	—
<i>E. coli</i> Crookes, extract	3	0.8	0.8	0.9	—
Rat-liver slices	3	2.7	1.7	1.6	1.2
Rabbit-liver homogenate	4	1.9	2.5	2.7	2.5
<i>E. coli</i> B	4	Trace	Trace	Trace	Trace

Table 2. *Recovery of added glutamate*

All listed results represent the average of at least duplicate complete experiments. For conditions see Methods.

Enzyme source	Glutamate		
	Added (μ moles)	Recovered	
		(μ moles)	(%)
Manometric (Method no. 4)			
None	3.0	2.2	73
	3.0	2.5	83
	3.0	3.0	100
	4.0	4.0	100
	6.2	5.1	82
	6.2	5.2	84
	6.2	6.8	110
	8.0	7.2	90
Rabbit-liver homogenate	3.0	2.8	93
	3.0	2.6	87
	6.0	5.4	90
	6.4	5.4	84
Rabbit, boiled-liver homogenate	6.0	5.5	91
<i>E. coli</i> B, boiled-cell suspension	4.0	3.4	85
	8.0	6.8	85
Chromatographic (Method no. 3)			
<i>E. coli</i> B, suspension	25	27	108
	25	26	104
<i>E. coli</i> B, extract	25	25	100
<i>E. coli</i> Crookes, suspension	25	22	88
	25	26	104
<i>E. coli</i> Crookes, extract	25	29	116
Rat-liver slices	4.0	5.0	125
	8.0	7.2	90
None	4.0	3.6	90
	4.0	3.8	95
	4.0	3.9	98
<i>E. coli</i> Crookes, suspension	4.0	2.5	63
	4.0	2.5	63
	4.0	2.6	65
None	8.0	8.5	106
	8.0	8.1	101
	8.0	8.0	100
<i>E. coli</i> Crookes, suspension	8.0	7.4	93
	8.0	6.8	85
	8.0	7.1	89

presence of the bacterial extract or suspension, ranging from 0.6 to 1.5 μ moles, represent the maximum limiting amounts that could be formed by transamination and yet escape detection.

DISCUSSION

The failure to confirm the results of Gunsalus & Tonzetich (1952), who reported as much as 6 μ moles of glutamate formed by transamination between amino purines and amino pyrimidines and α -oxo-glutarate, may stem from a variety of causes, among them differences in bacterial strains and composition of the culture media, growth and

harvesting conditions, techniques of measuring glutamate, etc. We endeavoured to obtain *E. coli* strains B and the Crookes strain from the original Laboratory (Cornell University); however, our bacterial preparations were ultimately derived from slants kindly sent to us from Illinois by Dr I. C. Gunsalus. We have no certain knowledge that either our strains or our culture media were identical with those employed by Gunsalus & Tonzetich (1952). The procedures for growth and harvesting are fairly routine operations; minor variations are not usually considered capable of producing qualitative changes in bacterial-enzyme content.

Our later procedures for estimating glutamate were based on elution from paper and spectrophotometric determination of the chromogen produced by the reaction between glutamate and ninhydrin; also glutamate was quite independently and specifically estimated by a manometric technique. These methods are in contrast to visual comparison of glutamate on ninhydrin-treated paper chromatograms (Gunsalus & Tonzetich, 1952).

A theoretical difference in our approach lay in the early appreciation that transamination between amino purines and pyrimidines and α -oxoglutarate, if it occurs at all, must be a somewhat different reaction from that commonly taking place between a primary amine and a carbonyl derivative. Thus transamination between an α -amino acid and an α -keto acid is usually represented as occurring in

a number of steps (Fig. 1, Scheme 1). A Schiff's base is formed by condensation of the amino and the keto acid. The α -hydrogen (proton) of the amino donor becomes labile in the presence of the keto acid (Braunstein, 1947). Actually this hydrogen may dissociate even under conditions excluding any transamination (Sprinson & Rittenberg, 1951). Once the Schiff's base is formed, however, both dissociation of the α -proton and an electromeric shift in the methylene-azomethine bridge take place. Simultaneously, a proton is taken up from the medium in the α -position of the original carbonyl compounds. Hydrolysis of the transformed Schiff's base results in a new keto acid corresponding to the original amino acid and an amino acid corresponding to the original keto acid. It should be noted that transamination involves oxidation of the amino to a keto acid, and the reduction of the

Scheme 1

Scheme 2

Fig. 1. Reaction schemes for transamination.

keto to an amino acid. The intramolecular oxidation-reductions or reversible prototropic change in the methylene-azomethine bridge (interconversion of the two Schiff's bases) is thought to be the enzyme-catalysed step. In his review, Braunstein (1947) states: 'It appears that dissociation of an alpha hydrogen is an independent, relatively non-specific and heat-resistant function of aminopherase, prerequisite for...transfer of amino groups (and electrons), i.e. by transamination proper.'

More elaborate schemes involving pyridoxal-pyridoxamine (Meister, 1955; Tannenbaum, 1956), while undoubtedly representing a more valid picture of what actually happens, have not contributed a clearer understanding of the fundamental mechanism of transamination. Examination of such a scheme (Fig. 1, Scheme 2) in which pyridoxal-pyridoxamine is included reveals that the original amino and keto acids do not directly interact, but that the amino group is transferred via the pyridoxal-pyridoxamine system again through Schiff's base-type compounds. Implicit, also, is the labilization of a proton of the amino methyl group of pyridoxamine in the usual fashion during transamination.

With adenine or cytosine, neither of the tautomeric structures (containing an amino or imino group respectively) has a proton at C-6. Thus an intramolecular oxidation-reduction cannot take place in these compounds similar to that occurring when amino acids act as amino donors in transamination. Dr A. Meister (personal correspondence) was kind enough to call our attention to a recent report by Mardashev & Pavlova (1955), describing transamination between amino purines and glyoxylic or glycollic acids. It would be difficult to reconcile such transfers of amino groups with present mechanisms proposed for transamination.

The tautomeric form which contains an imino group at C-6 is presumably not capable of undergoing transamination. Inspection of a current list of compounds which are reputed to engage in transamination fails to reveal the presence of an imino compound (Meister, 1955).

It would seem therefore that a prior reduction or opening of the pyrimidine ring is an obligatory prerequisite for transamination in adenine and cytosine. For this reason we included a common biological reducing agent, DPNH, in many of our test solutions. There was no evidence that such inclusion altered any experimental results.

No attempt was made to follow intrinsic purine or pyrimidine changes. It was felt that interpretation of such data would be complex since specific purine and pyrimidine deaminases and nucleoside deaminases are usually active in bacteria (Stephenson & Trim, 1938).

The results are fairly conclusive in that no transamination could be demonstrated between adenine, cytosine, adenosine or cytidine and α -oxoglutarate when *E. coli* strain B, *E. coli* strain Crookes, rabbit-liver homogenate or rat-liver slices were used as possible enzyme sources. However, with the bacteria small amounts of glutamic acid (approx. 1 μ mole) could have been formed by transamination and not have been measured. This is the amount of glutamate that could not be accounted for in recovery experiments when 4 or 8 μ moles of glutamate were incubated with *E. coli*, Crookes strain. Isotopically labelled compounds could conceivably be of aid in resolving the question whether transamination occurs to a very slight extent. We intend to investigate this possibility in the future.

Compounds other than α -amino and keto acids are known to participate in biological-transamination reactions. Indeed the basic requirements are reputed to be an unsubstituted amine, a carbonyl group, enzyme and pyridoxal or pyridoxamine. Another requirement, not specifically mentioned but suggested by examination of the list of compounds which may participate in transamination (Meister, 1955), is a proton on the carbon linked to the free amino group.

SUMMARY

1. No transamination could be demonstrated between α -oxoglutarate and adenine, adenosine, cytosine or cytidine, with *Escherichia coli* (strains B and Crookes), rabbit-liver homogenate and rat-liver slices as enzyme sources.

2. Analytical limitations and the possibility of slight metabolic utilization of formed glutamate by the bacteria renders equivocal the conclusion that transamination of adenine, cytosine and their ribosides is altogether absent, but the existence of a transamination reaction for these compounds must be sought by other techniques.

Pyridoxal phosphate was a gift from Dr W. Umbreit of the Merck Institute for Therapeutic Research.

We are indebted to Dr I. C. Gunsalus for sending us slants of *E. coli* (strains B and Crookes).

We are indebted to Dr Philip Cohen for sending us a flow sheet for the preparation of A.T.C. no. 4157. We also followed his suggestions for the manometric determination of glutamate. We also wish to thank Mrs S. Zehl who carried out some of the analyses for glutamate.

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The Hydrolysis of Indoxyl Esters by Rat Esterases

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It has been reported previously that human blood contains five esterases capable of hydrolysing unsubstituted indoxyl esters (Underhay, 1957). Three of these esterases, acetylcholinesterase, an aliphatic esterase and an aromatic esterase, are found in the erythrocytes and the remaining two esterases, butyrylcholinesterase and an aromatic esterase, which is not identical with the aromatic esterase of the erythrocytes, are present in plasma. When either human erythrocytes or human plasma are used as enzyme source, hydrolysis is mainly accomplished by the cholinesterases and the other esterases participate only to a small extent. In this paper, for reasons to be discussed below, the term 'aliphatic esterase' is replaced by 'organophosphate-sensitive esterase' and the term 'aromatic esterase' by 'organophosphate-resistant esterase'.

Most of the histochemical studies in which indoxyl esters have been used as substrates have so far been carried out on rat tissues (Holt, 1954, 1956). Experiments were therefore carried out to study the hydrolysis of unsubstituted indoxyl esters by erythrocyte preparations and plasma of the rat and by homogenates of rat liver from which the nuclei had been removed. The results obtained show that the hydrolysis of these esters by the three rat-enzyme preparations is mainly accomplished by organophosphate-sensitive esterases and that the cholinesterases and organophosphate-resistant esterases contribute to a lesser extent.

MATERIALS AND METHODS

Substrates. The substrates used were acetylcholine chloride, propionyl- and butyryl-choline iodides, indoxyl acetate, propionate and butyrate and α -naphthyl acetate. All were of analytical purity. Acetylcholine chloride was purchased from Roche Products Ltd. and the other substrates were kindly provided by Dr S. J. Holt.

Inhibitors. Inhibitors used were eserine sulphate, 3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methyl sulphate (Ro 3-0422), diethyl *p*-nitrophenyl phosphate (E 600), diisopropyl *p*-nitrophenyl phosphate (DINP), diisopropyl phosphorofluoridate (DFP) and the selective anti-acetylcholinesterase 3-oxo-1:5-diphenylpentane-*pp'*-bis(propyldimethylammonium) dibromide (B.W. 285 C51).

Enzyme preparations. Male white Wistar rats were used for all experiments. Blood was rapidly withdrawn by cardiac puncture under ether anaesthesia and collected in a tube containing heparin. The plasma was pipetted off after the blood had been centrifuged for 10 min. at 3700 rev./min. in an International Centrifuge, size 1, Type SB, and the erythrocytes were twice washed with 0.9% NaCl. Since the erythrocytes agglutinate rapidly on standing they were immediately haemolysed with 9 vol. of 0.025 M-NaHCO₃. Throughout the text the terms 'erythrocyte preparation' and 'plasma' refer to preparations obtained in this way. Zajicek & Datta (1952, 1953) and Hines (1952) have shown that platelets and white cells of rats also contain acetylcholinesterase. To investigate the effect of these components on the activity of the enzyme preparations described above, the following experiments were carried out. A cell count of one of the plasma samples showed that the number of platelets and white cells present was negligible; hydrolysis resulting from this contamination is too small to be recorded under the experimental conditions and more than 99% of the hydrolysis obtained with plasma as enzyme source is due to enzymes present in the plasma itself.

A cell count of two samples taken from one of the erythrocyte pellets showed that although both white cells and platelets were present they were far more numerous in the sample taken from the top than in that taken from the bottom of the pellet. Table 1 gives the rates of hydrolysis of acetylcholine and indoxyl acetate by the two samples together with a count of the cell types present. Also included are the rates of hydrolysis of both substrates by two different preparations of platelets. These results show that the platelets contribute approximately 30% to the hydrolysis of acetylcholine when erythrocyte preparations are used as enzyme source. With indoxyl acetate as substrate their contribution does not amount to more than 10% of the total hydrolysis. Inhibition studies with a 10^{-7} M-concentra-