# ADENYLATE KINASE OF MAMMALIAN ERYTHROCYTES

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SUMMARY

The various blood constituents have been shown to be devoid of adenylate kinase activity, with the exception of the erythrocytes, where the enzyme activity is not bound to the stromal fraction. The optimal $H^+$ concentration is at pH 7.5. Strong $Mg^{++}$ activation and rapid denaturation by heat were found, while no appreciable inhibition by $NaN_3$ was observed. The enzyme does not sediment when centrifuged for 210 min at 170,000 $\times$ *g*.

The preparation contains an enzyme that specifically catalyzes the reversible transformation 2 ADP $\rightleftharpoons$ ATP + AMP and is devoid of other appreciable nucleotide monophosphate kinase, ATPase and ADPase activity.

## INTRODUCTION

Studies on phosphorus metabolism have revealed the presence in red cells of the enzymes of the glycolytic pathway leading to adenosinetriphosphate synthesis[1] and several ATPases[2]. Similar enzymes have been described in leucocytes[3], and ATPase has been found in blood platelets[4].

Although adenylate kinase activity (2 ADP $\rightleftharpoons$ ATP + AMP) has been reported in human erythrocytes by OVERGAARD HANSEN[5], a thorough study of this enzyme, either in whole blood or in its separated constituents, has never been made.

A study of adenylate kinase activity of blood consituents and some properties of the enzyme are reported here.

## EXPERIMENTAL

### *Materials and methods*

Chromatographically pure ATP and ADP were prepared from the commercial products by chromatography on Dowex 1-X2, 200–400 mesh, according to COHN AND CARTER[6]. The eluted substances were freed from inorganic salts by adsorption on activated Norite and elution with $H_2O$–ethanol–$NH_3$ (54:45:1) as previously described[7]. Ammonia, alcohol, and part of the water were removed by concentrating *in vacuo*.

The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine 5'-monophosphate; ITP, inosine triphosphate; IDP, inosine diphosphate; IMP, inosine monophosphate; $P_I$, inorganic orthophosphate; Hb, hemoglobin; TCA, trichloroacetic acid.

ITP, IDP, IMP were the crystalline Na salts, kindly donated by Sigma. IDP was completely purified by ion exchange chromatography according to DEUTSCH AND NILSSON[8] and adsorption–elution on Norite[7].

Unless otherwise stated, the reaction mixture contained in a final volume of 0.5 ml, 2 m*M* ADP, 0.014 *M* diethylbarbiturate buffer of the desired pH, and an amount of the enzyme preparation measured by its Hb or protein content. The ratio of Hb (measured by the absorption of HbCO at 570 m$\mu$) to protein measured by the biuret reaction[9] was usually 5:6.

The reaction was started by adding the enzyme preparation to the incubation medium, equilibrated at 37°. It was carried out for 8 min at 37° under constant agitation in a thermostated bath and was stopped either by heating for 2 min at 100° in boiling water or by addition of 0.1 vol. 50 % TCA. The pH was checked before and after incubation. All experiments were carried out in triplicate. The samples were centrifuged and 0.1 ml of the clear supernatant was analyzed by descending paper chromatography in *n*-propanol–$H_2O$–100 % TCA–$NH_3$ (75:20:5:0.5)[10], the separated compounds being determined by the orcine reaction after elution, as previously described[10].

Controls to which no substrate or enzyme had been added did not show formation of either ATP or AMP.

*Enzyme preparations*

Whole human blood was collected in four volumes of isotonic NaCl in order to prevent immediate clotting. Platelets were prepared according to DILLARD *et al.*[11]. Red and white cells were sedimented from diluted blood at 1,500 × *g* and washed three times with isotonic NaCl. By suspending in bidistilled water to about 12 % Hb, hemolysis was produced and leucocytes were sedimented at 1,500 × *g* for 20 min. After further dilution to 5 % Hb, the stromal fraction was sedimented at 18,000 × *g* for 45 min, separated from the supernatant "hemolysate", and washed twice with water. A cytological control confirmed the homogeneity of each preparation. Serum was obtained from undiluted blood after clotting.

## RESULTS

The various blood constituents were assayed separately after adenylate kinase activity had been found in whole blood (10 mg Hb per samples). Reaction mixtures contained 12 mg protein for assays on white cells, platelets and stromas of erythrocytes and 4 mg for serum.

No activity was shown by either of these preparations; adenylate kinase activity was found, however, in hemolysed total erythrocytes (10 mg Hb per sample) and in the "hemolysate". Stromal free "hemolysate" was, therefore, used in all further experiments. Adenylate kinase activity, comparable with that of human cells, was also found in horse, pig and ox erythrocytes.

*Purity of the preparation*

In the following sections the results are reported as $\mu$moles of ATP and AMP formed, and $\mu$moles of residual ADP. It will be seen that the amounts of ATP and AMP do not always strictly coincide, as the stoichiometry of the reaction would

require. Differences are not large, however, and the recovery (ATP + AMP + ADP) always averages 100%. ATPase and ADPase were assayed in 0.014 *M* diethylbarbiturate buffer on 2 m*M* ATP or ADP at pH 8.5 with 5 mg Hb and at pH 7.6 with 0.5 mg Hb and $9.5 \cdot 10^{-4}$ *M* $Mg^{++}$. No $P_i$ was detected when directly assayed[12], and chromatography confirmed that there was no breakdown of ATP and only adenylate kinase activity was detectable on ADP. Nucleotide monophosphate kinase activity was assayed in 0.014 *M* diethylbarbiturate buffer pH 7.6 and $9.5 \cdot 10^{-4}$ M$MgCl_2$ on 1 m*M* ITP and IMP, ITP and AMP, ATP and IMP, and on 2 m*M* IDP, using 0.5 mg Hb on a final volume of 0.5 ml. No phosphate transfer or dephosphorylation were detected by chromatographic analysis[10].

Thus, the homogeneity of our preparation as regards enzymes acting on adenosine or inosine nucleotides seems rather satisfactory, since, as will be seen from subsequent sections, glycolytic and oxidative phosphorylations, ATP–$P_i$ exchange and deamination of ATP and ADP can also be excluded.

*Reversibility of the reaction*

The reversible interconversion 2 ADP $\rightleftharpoons$ ATP + AMP was demonstrated by parallel experiments on samples containing either ADP or ATP + AMP as substrate. Conditions and results are shown in Table I.

TABLE I

REVERSIBILITY OF THE ADENYLATE KINASE REACTION IN HUMAN ERYTHROCYTES

Assay medium: 0.014 *M* diethylbarbiturate buffer pH 7.4, $9.5 \cdot 10^{-4}$ *M* $MgCl_2$, substrate see below, 0.1 ml hemolysate containing 0.5 mg Hb. Final volume 0.5 ml; 8 min at 38°; reaction stopped by addition of 0.1 vol. 50% TCA.

| *Substrate* | *Formed (μmoles)* | *Residual (μmoles)* | *Total recovery (μmoles)* |
|---|---|---|---|
| 1 μmole ADP | ATP 0.15<br>AMP 0.19 | ADP 0.65 | 0.99 |
| 0.5 μmole ATP<br>+ 0.5 μmole AMP | ADP 0.40 | ATP 0.30<br>AMP 0.31 | 1.01 |

*pH optimum*

The pH/activity curve is shown in Fig. 1. A clear optimum is found at pH 7.4–7.6.

When greater amounts of enzyme preparations were used (above 10 mg Hb), formation of IMP was observed from pH 6 to pH 7.4. No other adenine or inosine nucleotides or nucleosides or free purines could be detected at any pH.

*Thermal stability*

The inactivation of the enzyme by heat was studied by adding a volume of the hemolysate to twice its volume of water, previously equilibrated at the desired temperature in a water bath. The heating was continued under constant agitation, and aliquots were taken at regular intervals. These were immediately cooled on a refrigerated plate and subsequently used for determining the residual activity. Results are shown in Table II. Preparations heated in 0.1 *N* HCl were compared with an unheated hemolysate 0.1 *N* with respect to HCl.

Heating at 100° in water (pH 6) or in 0.1 *N* HCl completely destroys the enzyme activity in 2 min, while on heating at 50° in water about 50 % of the original activity is retained even after 30 min preincubation.

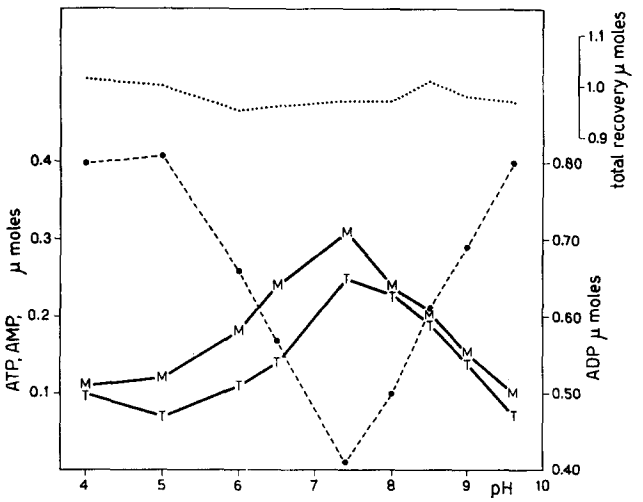


Fig. 1. pH/activity curve of erythrocyte adenylate kinase. 2 m*M* ADP, 0.014 *M* diethylbarbiturate buffer of the desired pH, 0.1 ml hemolysate containing 3 mg Hb. Final volume 0.5 ml; 8 min at 38°; reaction stopped by addition of 0.1 vol. 100 % TCA. ——— μmoles ATP (T) and AMP (M) formed; – – – – μmoles ADP residual; ····· total recovery in μmoles.

TABLE II

THERMAL INACTIVATION OF HUMAN ERYTHROCYTE ADENYLATE KINASE

Assay medium: 0.014 *M* diethylbarbiturate buffer pH 7.6, 1 μmole ADP, 0.1 ml hemolysate containing 5 mg Hb. Final volume 0.5 ml; 8 min at 38°; reaction stopped by addition of 0.1 vol. 50 % TCA.

| *Preheating at 50°* (*minutes*) | *Formed* (*μmoles*) | | *Residual* (*μmoles*) | *Total recovery* (*μmoles*) |
|---|---|---|---|---|
| | *ATP* | *AMP* | *ADP* | |
| 0 | 0.24 | 0.22 | 0.50 | 0.96 |
| 5 | 0.13 | 0.16 | 0.74 | 1.03 |
| 15 | 0.11 | 0.15 | 0.74 | 1.00 |
| 30 | 0.12 | 0.15 | 0.72 | 0.99 |

*Activators and inhibitors*

The effect of increasing $Mg^{++}$ concentration was determined on samples containing an amount of enzyme preparation corresponding to 0.66 mg Hb. The activity increases gradually: at optimum concentration ($9.5 \cdot 10^{-4}$ *M* $MgCl_2$) it is six times as great as when no $MgCl_2$ is added. At higher concentrations (maximum $MgCl_2$ concentration assayed $3.6 \cdot 10^{-3}$ *M*) it is slightly less than the optimum.

The effect of 0.002 *M* $NaN_3$ was studied under the conditions described in a previous section for ATPase and ADPase activity. A decrease of ATP formation is observed; it averages 25 % at pH 8.5, 3 mg Hb, no $MgCl_2$ added, and 18 % at pH 7.6, $9.5 \cdot 10^{-4}$ *M* $MgCl_2$, 0.5 mg Hb.

*Effect of different buffers*

Activities in 0.03 *M*, 0.014 *M* mono and disodium phosphate, 0.014 *M* diethylbarbiturate and tris buffers pH 7.6 were compared at optimum $Mg^{++}$ concentration and 0.5 mg Hb. With respect to diethylbarbiturate, the ATP/AMP ratio increases by 5 % in 0.014 *M* phosphate, decreases by 16 % in 0.03 *M* phosphate and by 34 % in trishydroxymethylaminomethane. In order to ascertain whether $P_i$ was added to ADP in other ways than by adenylate kinase activity, the experiment with 0.014 *M* phosphate buffer was repeated with addition of 0.1 $\mu$C $^{32}P_i$.

No labelling was found in ATP, ADP or AMP, either by direct counting or by autoradiography. These results rule out the possibility, under our experimental conditions, of ATP formation in the glycolytic or oxidative pathway, or an ATP $\rightleftharpoons P_i$ exchange.

*Ultracentrifugation*

The hemolysate was centrifuged for 210 min at 39,500 rev./min in a Spinco Model L ultracentrifuge, swinging rotor SW 39. No boundaries were observed in the centrifuged fluid: the upper portion of supernatant (1 ml, average centrifugal force 90,000 $\times$ *g*) was collected separately from the rest (170,000 $\times$ *g* at bottom). A very small precipitate (P) was obtained and assayed after suspension in water without washings. Each sample contained 0.6 mg protein and $9.5 \cdot 10^{-4}$ *M* $MgCl_2$.

The specific activities of both fractions of the supernatant are the same as those of the uncentrifuged hemolysate, while in the precipitate only ADPase activity is found. Since stromas had been centrifuged off the hemolysate used for this experiment at 15,500 $\times$ *g* for 30 min, it is likely that fraction P is due to unsedimented stromal residues.

## DISCUSSION

Like myokinase, the adenylate kinase of erythrocytes is specific for the reaction 2 ADP $\rightleftharpoons$ AMP + ADP.

BOWEN AND KERWIN[13] found that the optimum of resin-purified muscle enzyme is about pH 8 and that it is shifted to lower values by $Mg^{++}$ concentrations higher than $3 \cdot 10^{-4}$ *M* ([Mg] to [ADP] ratio = 0.6). In our experiments, the medium used for determining the pH/activity curve was probably about $4 \cdot 10^{-5}$ *M* with respect to $Mg^{++}$ owing to the endocellular Mg[14] dissolved in the hemolysate ([Mg] to [ADP] ratio = 0.02): it seems very unlikely that this Mg concentration would influence the pH optimum.

The activation by $Mg^{++}$ is also much greater than that found for myokinase by BOWEN AND KERWIN[13]. They observed that the heat stability of the muscle enzyme decreases during purification; $Mg^{++}$ $10^{-6}$ and $10^{-3}$ *M*, NaCl, albumin[15] and actomyosin[16] partially protect the enzyme against heat inactivation. Our data are consistent with those of BARGONI[15] on the protective effect of $Mg^{++}$ on myokinase; they show that Hb does not have the protective effect on the enzyme of erythrocytes that it has on myokinase[17]. The results of ultracentrifugation suggest that the adenylate kinase of erythrocytes has a relatively low molecular weight (crystalline myokinase has a molecular weight of 21,000[18]). In the light of these findings, it is reasonable to suppose that in the "hemolysate" adenylate kinase is found in a higher degree of purity than is the enzyme of unpurified muscle extracts.

ACKNOWLEDGEMENT

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REFERENCES

1 T. A. J. PRANKERD AND K. I. ALTMAN, *Biochem. J.*, 58 (1954) 622.
2 T. V. VENKSTERN AND V. A. ENGELHARDT, *Biochimiya*, 22 (1957) 911 (English transl. 855).
3 W. S. BECK, *Ann. N.Y. Acad. Sci.*, 75 (1958) 4.
4 A. PEDRAZZINI AND E. SALVIDIO, *Acta Haematol.*, 18 (1957) 42.
5 K. OVERGAARD HANSEN, *Acta Pharmacol. Toxicol.*, 14 (1957) 67.
6 W. E. COHN AND C. E. CARTER, *J. Am. Chem. Soc.*, 72 (1950) 4273.
7 P. CERLETTI, M. L. AEBISCHER AND C. FRONTICELLI, *Ricerca sci.*, 28 (1958) 2052.
8 A. DEUTSCH AND R. NILSON, *Acta Chem. Scand.*, 7 (1953) 1288.
9 J. W. MEHL, *J. Biol. Chem.*, 157 (1944) 173.
10 P. CERLETTI, P. L. IPATA AND N. SILIPRANDI, *Anal. Chim. Acta*, 16 (1957) 548.
11 G. H. C. DILLARD, G. BRECKER AND E. P. CRONKITE, *Proc. Soc. Exptl. Biol. Med.*, 78 (1951) 853.
12 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
13 W. J. BOWEN AND T. D. KERWIN, *Arch. Biochem. Biophys.*, 64 (1956) 278.
14 E. ERDOE AND I. A. MIRSKY, in W. S. SPECTOR, *Handbook of Biological Data*, W. B. Saunders Co., Philadelphia and London, 1956, p. 52.
15 N. BARGONI, *Z. physiol. Chem.*, 311 (1958) 173.
16 A. SZENT-GYÖRGYI, *Chemistry of Muscular Contraction*, 2nd edn., Academic Press, New York, 1951.
17 O. H. CALLAGHAN, *Biochem. J.*, 66 (1957) 7P.
18 L. NODA AND S. A. KUBY, *J. Biol. Chem.*, 226 (1957) 541.

*Biochim. Biophys. Acta*, 38 (1960) 45 50

# AN EFFECT OF THE SUBSTRATE ON UREA-MODIFIED PENICILLINASE

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SUMMARY

α-Penicillinase retains full activity when exposed simultaneously to the substrate and to 0.0025 *M* iodine. Urea-modified (γ-type) penicillinase is inactivated under such conditions. Preincubation with the substrate causes a shift from γ- to α-type, as measured by change in reactivity with iodine. The shift is not reflected in the rate of the enzymic reaction. The significance of the substrate-induced change is discussed.

## INTRODUCTION

Exopenicillinase of *Bacillus cereus* can exist in two antigenically different states, which also differ in their reactivity towards iodine[1]. The transition from one state to

the other can be brought about in a number of ways, including exposure to hydrogen-bond breaking agents, such as urea[2] or guanidine hydrochloride[3]. The resulting modification of penicillinase does not, however, seem to affect its enzymic activity. Thus the molecular activities of the original ($\alpha$-type) and of the urea-modified ($\gamma$-type) enzyme are practically identical[2].

It appeared at first as if the properties examined reflected configurational changes not involving regions the integrity of which was essential for enzymic function[1]. Yet, though not impossible, it was difficult to visualize a situation where treatment known to break hydrogen bonds and shown to result in exposure of previously unreactive groups would leave the enzymic activity of the molecule completely unimpaired. We therefore suggested an alternative interpretation, whereby the enzyme molecule is restored to the original $\alpha$-configuration through contact with the substrate[2].

The behaviour of urea-modified penicillinase in the presence of the substrate has now been examined and the data to be presented seem to favour the latter interpretation.

## MATERIALS AND METHODS

*$\alpha$-penicillinase.* A preparation of penicillinase was obtained from the culture supernatant of *Bacillus cereus*, mutant strain 569/H. This mutant strain, derived[4] from the inducible penicillinase forming *Bacillus cereus* NRRL 569, produces penicillinase constitutively. The culture was started from spores inoculated into a medium consisting of Difco Peptone (1 %) Bovril Meat Extract (0.3 %) and NaCl (0.2 %) and prepared according to POLLOCK[5].

After reaching logarithmic phase the cells were transferred to a casein–hydrolysate–citrate medium (CH/C) prepared according to KOGUT *et al.*[4] and shaken at 35° until enzyme production began to level off. The supernatant enzyme was concentrated by adsorption and purified by a procedure described elsewhere[3] or by the method of KOGUT *et al.*[4]. In either case the specific activity of the penicillinase preparation used was close to that found for the crystalline preparation of $\alpha$-penicillinase[4] of *B. cereus* 569. Such preparation will be referred to as $\alpha$-penicillinase. It was stored at —5°, and all dilutions were made in 0.5 % gelatin unless otherwise stated.

*Urea-modified penicillinase.* As shown previously[2], $\alpha$-penicillinase becomes sensitive to 0.0025 *M* iodine if preincubated with 5 *M* urea. Preincubation is not necessary if gelatin is omitted from the system, and most of the enzyme is instantaneously inactivated by the iodine in an aqueous solution of urea[3]. Thus penicillinase assayed under conditions in which the gelatin solution has been replaced by a 5 *M* urea solution will be referred to as urea-modified penicillinase.

*Penicillin and penicilloic acid.* The penicillin used was the sodium salt of benzylpenicillin (Merck). Penicilloic acid was prepared by alkaline cleavage of penicillin[6]. 1 ml of a solution of benzylpenicillin, containing 200,000 units, was treated with 1 drop of 20 % NaOH for 20 min at room temperature. It was then diluted 1:40 in 0.1 *M* phosphate buffer pH 7. Such a preparation was found to bind an amount of iodine equivalent to that bound by an enzymically cleaved preparation.

*Enzyme assay.* The iodometric procedure for assay of $\alpha$-penicillinase has been described[1,3]. In that procedure the $\alpha$-type activity is determined by the time required for a complete decolorization of a known amount of iodine present in the assay

mixture. The decolorization is due to the formation of a colourless complex between the iodine and the reaction product (penicilloic acid). The iodine concentration at the beginning of the reaction is sufficient to inactivate completely the immunologically distinct $\gamma$-penicillinase[7]. It also inactivates reversibly modified penicillinase, said to be in the $\gamma$-state[1,2].

Modifications of the standard procedure will be dealt with in the next section.

## RESULTS

### *Assay of α-penicillinase*

A. *Variation of time of iodine addition.* The following procedure has been adopted for assaying changes in the iodine-sensitivity of penicillinase in the course of the enzyme reaction. The reaction is started by introducing 150–200 units of penicillinase in 0.2 ml into an assay mixture which consists of 3 ml of the assay medium, *e.g.* 0.5 % gelatin solution, and 1 ml of 0.1 *M* phosphate buffer pH 7 containing 5000 units of penicillin. 0.5 ml of iodine solution of 0.025 *M* $I_2$ in 0.125 *M* KI aqueous solution is added after a specified time interval. The time required for a complete decolorization is noted, and the activity calculated as in the standard procedure[1]. It will be realized that since the penicilloic acid formed combines instantaneously with its equivalent of iodine, the end point of the decolorization in the case of α-penicillinase should be independent of the time of addition of iodine.

The first set of measurements consisted of assays of α-penicillinase in 0.5 % gelatin with the iodine added at 30 sec intervals. As can be seen from Table I (columns

TABLE I

ASSAY OF α-PENICILLINASE WITH VARYING TIMES OF ADDITION OF IODINE

| *A* *Time before iodine addition (min)* | *B* *Decolorization time (sec)* | *C* *Activity (units/ml)* | *D* *Deviation from mean value* % |
|---|---|---|---|
| 0.0 | 311 | 187.5 | +5.6 |
| 0.5 | 369 | 160.0 | —9.8 |
| 1.0 | 360 | 161.3 | —9.1 |
| 1.5 | 342 | 172.5 | —2.8 |
| 2.0 | 312 | 187.0 | +5.4 |
| 2.5 | 310 | 187.5 | +5.6 |
| 3.0 | 300 | 194.0 | +9.4 |
| 3.5 | 311 | 187.5 | +5.6 |
| 4.0 | 314 | 186.3 | +5.0 |
| 4.5 | 344 | 170.5 | —3.9 |
| 5.0 | 352 | 166.8 | —6.0 |

A, B) the decolorization time is in fact independent of the time of addition of iodine. Columns C, D (Table I) demonstrate that the accuracy of the iodometric assay is not impaired by the procedure of adding iodine in the course of the reaction.

B. *Variation of iodine concentration.* A progressive delay in addition of iodine to the assay system should be in principle equivalent to the exposure of the enzyme to progressively decreasing concentrations of iodine. This is because before the addition of the iodine the enzyme produces an amount of penicilloic acid proportional

to the time of delay; penicilloic acid combines instantaneously with the added iodine and reduces its effective concentration to a value inversely proportional to the time of delay.

In order to test the validity of the iodometric procedure for various iodine concentrations a second series of readings was taken with iodine present at zero time. The variable in this set of assays was the initial concentration of iodine. Should $\alpha$-activity be unaffected by the iodine concentrations tested, the decolorization time would be directly proportional to the amount of iodine in the assay system. The results are summarized in Table II. By multiplying the time of decolorization by the factor of iodine dilution we obtain the time required for complete decolorization of the amount of iodine used in the standard assay. The corresponding activity is then read from the standard calibration curve.

It is obvious from Table II that the activity of $\alpha$-penicillinase is completely unaffected by an initial exposure to any concentration of iodine within the range tested.

TABLE II

ASSAY OF $\alpha$-PENICILLINASE WITH VARYING CONCENTRATIONS OF IODINE

| *A* *Iodine concentration* ($\times 10^{-4}$ *M*) | *B* *Decolorization time* (sec) | *C* $\left(\frac{B}{A} 25 \cdot 10^{-4}\right)$ * (sec) | *D* *Activity* (units/ml) | *E* *Deviation from mean value* % |
|---|---|---|---|---|
| 25.0 | 309 | 309 | 190.0 | −3.3 |
| 22.5 | 267 | 297 | 197.5 | +0.5 |
| 20.0 | 240 | 300 | 195.0 | −0.8 |
| 17.5 | 220 | 315 | 186.5 | −5.2 |
| 15.0 | 175 | 292 | 200.1 | +1.8 |
| 12.5 | 140 | 280 | 208.8 | +6.3 |
| 10.0 | 117 | 292 | 200.1 | +1.8 |
| 7.5 | 92 | 306 | 192.5 | −2.0 |
| 5.0 | 59 | 295 | 198.8 | +1.2 |

* Time required for decolorization of the amount of iodine used under standard assay conditions (values of column B divided by those of A and multiplied by the standard iodine concentration).

*The iodine sensitivity of penicillinase in urea*

It follows from the previous paragraph that $\alpha$-type activity is not affected by variations in the time of addition of iodine (set A) or in the initial concentration of iodine (set B).

In contrast, the activity of penicillinase in the $\gamma$-state is largely determined by these variables (see also ref. [8]). The question to be answered is whether the survival of $\gamma$-type activity as a function of exposure to iodine (sets A and B) is affected by previous contact with the substrate (set A).

The system studied here is that of penicillinase in urea. As shown previously[2], urea causes a shift from $\alpha$-type to $\gamma$-type activity. In the following experiments the enzyme was assayed for sensitivity to iodine by both procedures described above except that the gelatin solution used in the standard assay was replaced[3] by a urea solution of 5 *M*. The concentration of the enzyme was adjusted so that the amount of penicilloic acid formed in 30 sec would be approximately sufficient to combine with $2.5 \cdot 10^{-4}$ *M* iodine. This relationship is expressed in the paired abscissae of Fig. 1.

Again one set of readings was taken with varying concentrations of iodine in the assay system. After correcting for the differences in decolorization time due to the variable amounts of iodine, a curve has been obtained which simply describes the survival of penicillinase in urea as a function of the iodine concentration to which it was initially exposed (Fig. 1, curve B). This curve will be compared with the one obtained from the other set of readings, in which the full amount of iodine is added at various times after the initiation of the enzymic reaction (curve A in Fig. 1).

It will be noted that curve A too represents the activity of the enzyme after exposure to iodine. (The assay was started—as before—upon addition of iodine, and the reduction in decolorization time due to preformed penicilloic acid has been corrected for).

As there was no difference in the conditions of the assay, both enzyme survival curves should overlap, unless previous contact with the substrate affected the subsequent reactivity of the enzyme with iodine.

In our case the "iodine delay" curve (Fig. 1A) is significantly different from the "iodine concentration" curve (Fig. 1B). While curve B shows a nearly linear dependence of the activity on the concentration of iodine, curve A shows a sharp increase in the iodine resistance of the enzyme as the reaction proceeds. Thus the enzyme was invariably found to become fully resistant to iodine before the reaction was half over, *i.e.* at the stage where the effective iodine concentration is sufficient to inactivate approximately half of the enzyme in urea.

*Kinetics of the activity of penicillinase in urea*

As shown in Fig. 1, the enzyme in urea becomes increasingly more iodine-resistant as the reaction proceeds. The problem arose whether the enzymic activity of the preparation was in any way affected by this change in reactivity with iodine. As pointed out in the introduction, this could be answered in the negative by considering the fact that the sum of $\alpha$-type and $\gamma$-type activities was practically constant over a wide range of urea concentrations, and thus largely independent of the degree of the $\alpha$- to $\gamma$-conversion.

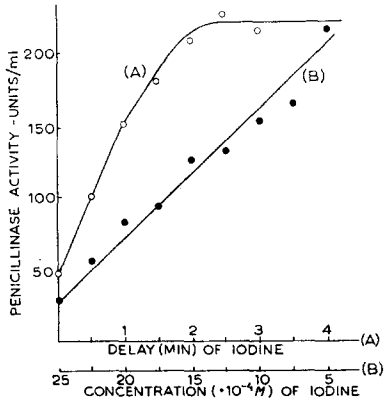


Fig. 1. Survival of penicillinase in urea: (A) as a function of time elapsed before addition of iodine. (B) as a function of the initial iodine concentration.

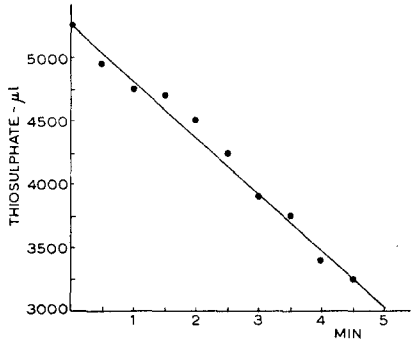


Fig. 2. Rate of penicilloic acid formation in the presence of urea. Iodine uptake by penicilloic acid formed in the course of the enzymic reaction determined by thiosulphate titration of residual iodine.

For a direct answer the actual rate of penicilloic acid formation in the presence of urea was determined. The assay of penicilloic acid was performed by a modification of a procedure suggested by PERRET[9].

A preparation of penicillinase in urea was allowed to react with the substrate under conditions identical with those of the "delayed iodine" assay. The reaction was stopped by the addition of 0.5 ml of a reagent containing 12.8 *M* acetic acid and 0.05 *M* iodine followed by 10 min incubation at 30°. By that time the enzymic activity was completely stopped and the penicilloic acid formed before the addition of the acetate reagent has combined with part of the iodine. The residual iodine was titrated against 0.0166 *N* Na-thiosulphate and the amount of penicilloic acid calculated from the difference. The results show that the formation of penicilloic acid is linear throughout, irrespective of the state of iodine sensitivity of the enzyme (Fig. 2).

*Possible factors involved in the γ- to α-shift in the course of the enzymic reaction*

The observed shift in the iodine sensitivity of the enzyme in urea could be ascribed to one or more of the following factors:

(a) contact with penicillin; (b) exposure to the accumulated penicilloic acid; (c) exposure to the phosphate buffer in which penicillin is delivered.

Factors (b) and (c) have been eliminated as a likely cause for the shift on the following grounds:

*The effect of penicilloic acid.* Penicilloic acid was prepared by alkaline cleavage of Na-benzyl-penicillin (see MATERIALS AND METHODS).

The enzyme preparation was preincubated for 5 min in 5 *M* urea. An amount of penicilloic acid just sufficient to combine with $5 \cdot 10^{-4}$ *M* iodine was added to the enzyme, (a) 60 sec before the beginning of the assay, (b) 60 sec after the beginning of the assay.

The iodine was added in both cases 60 sec after the beginning of the assay. At that stage the γ-type activity still forms a high proportion of the total (*cf.* Fig. 1) so that a two-minute contact with the penicilloic acid should cause a very marked increase in the activity surviving the exposure to iodine, if penicilloic acid were responsible for the shift.

As the readings obtained under conditions (a) and (b) were practically identical, it was concluded that the contact with penicilloic acid does not affect the iodine reactivity of the enzyme.

*The effect of phosphate buffer.* In the above assay procedure penicillin is delivered in 1 ml of 0.1 *M* sodium–potassium phosphate buffer, the function of which is to prevent acidification of the assay medium. The following experiment served to separate the possible effect of the phosphate from that of the substrate.

Penicillin was dissolved in distilled water at a concentration of 50,000 U/ml. 0.1 ml of that solution was used in the "5 *M* urea" assay. The phosphate buffer was added (a) 60 sec before the beginning of the assay, (b) 60 sec after the beginning of the assay.

The iodine was added in both cases 60 sec after the beginning of the assay.

As explained in the previous paragraph, the conditions were such that any effect of contact with phosphate on the iodine-sensitivity of the enzyme would cause a difference in values obtained under assay conditions (a) and (b), and as before, no such difference appeared.

## DISCUSSION

In defining the changes observed in penicillinase-treated in a number of ways[1] we have adopted two independent criteria. One was the antigenic identity of the enzyme, as judged by its neutralizability by anti-$\alpha$-penicillinase serum. The other was its reactivity with iodine, as judged by loss of activity through exposure to 0.0025 *M* iodine. Serological observations are difficult to interpret in experiments involving high concentrations of urea, so that the experimental evidence to be discussed rests mainly on measurements of changes in the iodine-sensitivity of the enzyme. While the iodine-sensitivity has not been shown to be sufficient to define the state of penicillinase with respect to antigenicity, it will be realized that wherever definable (*e.g.* in case of penicillinase pretreated with alkali or variously adsorbed[1]) the antigenic structure has been found to parallel the state of iodine-sensitivity. We also know that urea-modified penicillinase spontaneously reverts to the $\alpha$-state, as judged by both criteria, when urea is removed[3].

We have shown that the actual iodine-sensitivity of penicillinase in urea (indicated by curve A in Fig. 1) deviates from the expected (Fig. 1, curve B) as the enzymic reaction proceeds. This deviation was found not to be due to contact with the penicilloic acid formed or with the phosphate buffer present. Thus by implication, it was through previous contact with the substrate that the enzyme acquired resistance to iodine.

Since we do not know of any other mechanism that would account for the observed changes, we suggest that the increase in iodine resistance in the course of the enzymic reaction represents a $\gamma$- to $\alpha$-shift.

It will be remembered in this connection that the cell of *B. cereus* offers surfaces which by adsorbing the enzyme confer on it the properties of reversible $\gamma$-penicillinase[1]. A possible significance of a substrate-catalysed $\gamma \rightarrow \alpha$ shift in the context of the mechanism of penicillinase induction has to be borne in mind.

The experiments reported above were mainly designed to account for the observation that penicillinase seems to undergo configurational changes without any apparent impairment of its enzymic function. In our previous communication[2] we suggested a situation not unlike that observed in the case of ribonuclease[10], where the modified molecule reverts to the enzymically active conformation through contact with the substrate. While not conclusive, the present results are consistent with such an interpretation.

## REFERENCES


1 N. CITRI, *Biochim. Biophys. Acta*, 27 (1958) 277.
2 N. CITRI AND N. GARBER, *Biochim. Biophys. Acta*, 30 (1958) 664.
3 N. CITRI, N. GARBER AND M. SELA, in preparation.
4 M. KOGUT, M. R. POLLOCK AND E. J. TRIDGELL, *Biochem. J.*, 62 (1956) 391.
5 M. R. POLLOCK, *J. Gen. Microbiol.*, 8 (1953) 186.
6 W. H. FLOREY *et al.*, in *Antibiotics*, Vol. II (1949) 839.
7 M. R. POLLOCK, *J. Gen. Microbiol.*, 15 (1956) 154.
8 N. CITRI AND R. TOLEDO, in preparation.
9 C. J. PERRET, *Nature*, 174 (1954) 1012.
10 M. SELA, C. B. ANFINSEN AND W. F. HARRINGTON, *Biochim. Biophys. Acta*, 26 (1957) 502.