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# MECHANISM OF INHIBITORY EFFECT OF SOME PYRAZOLE DERIVATIVES ON PURINE BIOSYNTHESIS *DE NOVO*

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**Abstract**—The inhibitory effect of some pyrazole derivatives on GAR synthesis was studied. It was found that nonalkylating analogues and chloroethyltriazeno analogues inhibited GAR synthesis. A possible mechanism of action of triazeno derivatives on purine biosynthesis *de novo* was shown.

In previous studies we found that some pyrazole analogues of 4(5)-aminoimidazole-5(4)-carboxamide inhibited purine biosynthesis *de novo* in the pigeon liver cell-free system [1]. It was demonstrated that unsubstituted at ring nitrogen analogues as 3-amino-4-carbethoxypyrazole (ACEP), 3-amino-4-carboxypyrazole (ACP) were more active in the cell-free system as well as on micro-organisms. The alkylating analogues 3-(3′,3′-bis-β-chloroethyl-1′-triazeno)-4-carbethoxypyrazole (bis-chloroethyltriazeno-CEP) displayed almost equal activities as ACEP, while on bacteria all triazenes were considerably more active than nonalkylating derivatives.

Some purine bases and nucleotides as well as their analogues are known to inhibit the first steps of purine biosynthesis up to FGAR (5′-phosphoribosyl-*N*-formylglycineamide, *N*-formylglycineamideribotide) formation. These steps are the first level where metabolic control of purine biosynthesis *de novo* takes place. In order to discover the main step inhibited by the compounds tested, we investigated them on pigeon liver cell-free system treated with ion-exchange resin to remove the folic acid derivatives. This allows us to stop purine biosynthesis up to FGAR synthesis.

Preussman *et al.* [2, 3] found that 1-aryl-3,3-dialkyltriazenes underwent enzymatic dealkylation by rat liver microsomal fraction in two steps: to corresponding monoalkyltriazenes and then to corresponding amines. Other authors have suggested another mechanism of alkylating action via diazonium salts [4–6]. To verify these hypotheses with our triazenes we tested several dialkyl- and bis-chloroethyltriazenopyrazoles and corresponding diazonium salts.

## MATERIALS AND METHODS

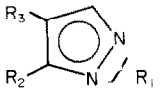
The inhibitors 3-amino-4-carbethoxypyrazole (ACEP), 3-amino-4-carboxypyrazole (ACP), *N*-hydroxyethyl-3(5)-amino-4-carbethoxypyrazole (*N*-hydroxyethyl-ACEP) were obtained by the method of Druey and Schmidt [7] and Schmidt *et al.* [8]. The triazenes 3-(3′,3′-bis-β-chloroethyl-1′-triazeno)-4-carbethoxypyrazole (bis-chloroethyltriazeno-CEP), *N*-methyl-3-(5)-(3′,3′-bis-β-chloroethyl-1′-triazeno)-4-carbethoxypyrazole (*N*-methyl-bis-chloroethyltriazeno-CEP), *N*-hydroxyethyl-3(5)-(3′3′-bis-β-chloroethyl-1′-triazeno)-4-carbethoxypyrazole (*N*-hydroxyethyl-bis-chloroethyltriazeno-CEP), 3(3′,3′-dimethyl-1′-triazeno)-4-carbethoxypyrazole (dimethyltriazeno-CEP), 3-(3′,3′-diethyl-1′-triazeno)-4-carbethoxypyrazole (diethyltriazeno-CEP), and *N*-hydroxyethyl-3(5)-(3′,3′-diethyl-1′-triazeno)-4-carbethoxypyrazole (*N*-hydroxyethyl-diethyltriazeno-CEP) were synthesized as described previously [9]. The diazonium salts 3-diazonium-4-carbethoxypyrazole (diazonium-CEP), *N*-methyl-3(5)-diazonium-4-carbethoxypyrazole (*N*-methyl-diazonium-CEP) and *N*-hydroxyethyl-3(5)-diazonium-4-carbethoxypyrazole (*N*-hydroxyethyl-diazonium-CEP) were synthesized by diazotizing the corresponding aminocarbethoxypyrazoles [10].

[$^{14}$C]Formate was purchased from Isocommerz, [1-$^{14}$C]glycine from Amersham, glutamine, ribose-5-phosphate, ATP, 3-phosphoglyceric acid, glycine from Sigma, D,L-homocysteine was obtained *ex tempore* [1]. All other substances used were analytical grade reagents.

The preparation of pigeon liver acetone powder, enzyme solution and the measuring *in vitro* incorporation of [$^{14}$C]formate into inosinic acid were described previously [1]. The incubation mixture contained in a final volume of 2.3 ml the following compounds in μmoles/ml: glutamine, 4.3; ATP, 2.2; ribose-5-phosphate, 2.2; 3-phosphoglycerate, 3.2; $MgCl_2$, 2.2; D,L-homocysteine, 2.2; glycine, 2.2; inhibitors, 4.4; 2.2; 1.5; 0.43; 0.22 resp.; veronal buffer, 22 (pH 7.5); boiled extract of pigeon liver, 0.20 ml; enzyme extract, 0.50 ml; [$^{14}$C]formate, 0.5 μCi.

The action of analogues on GAR (5′-phosphoribosylglycineamide, glycineamideribotide) synthesis was tested by measuring *in vitro* incorporation of [1-$^{14}$C]glycine into GAR using the technique of Goldthwait *et al.* [11]. The enzyme system was isolated before use by extraction of acetone powder with 0.05 M $KHCO_3$. The extract was passed through a Dowex-1 (bicarbonate form, 4 per cent cross linkage) column, dialyzed overnight against 0.05 M $K_2HPO_4$ and lyophilized. This enzyme preparation gave about 1 per cent incorporation of [1-$^{14}$C]glycine

Table 1. Inhibitory effect of pyrazole derivatives on GAR synthesis at 4 μmoles/ml



I

| Pyrazole derivatives | Inhibition | Number of exp. |
|---|---|---|
| $R_1 = H$, $R_2 = NH_2$, $R_3 = COOC_2H_5$ (ACEP) | 30 ± 2* | 3 |
| $R_1 = H$, $R_2 = NH_2$, $R_3 = COOH$ (ACP) | 6 ± 5 | 3 |
| $R_1 = CH_2CH_2OH$, $R_2 = NH_2$, $R_3 = COOC_2H_5$ (*N*-hydroxyethyl-ACEP) | 3 ± 2 | 3 |
| $R_1 = H$, $R_2 = N{=}N{-}N(CH_2CH_2Cl)_2$, $R_3 = COOC_2H_5$ (bis-chloroethyltriazeno-CEP) | 40 ± 3 | 4 |
| $R_1 = CH_3$, $R_2 = N{=}N{-}N(CH_2CH_2Cl)_2$, $R_3 = COOC_2H_5$ (*N*-methyl-bis-chloroethyltriazeno-CEP) | 11 ± 4 | 4 |
| $R_1 = CH_2CH_2OH$, $R_2 = N{=}N{-}N(CH_2CH_2Cl)_2$, $R_3 = COOC_2H_5$ (*N*-hydroxyethyl-bis-chloroethyltriazeno-CEP) | 10 ± 2 | 4 |
| $R_1 = H$, $R_2 = N{=}N{-}N(CH_3)_2$, $R_3 = COOC_2H_5$ (dimethyltriazeno-CEP) | 0† | 4 |
| $R_1 = H$, $R_2 = N{=}N{-}N(C_2H_5)_2$, $R_3 = COOC_2H_5$ (diethyltriazeno-CEP) | 0† | 4 |
| $R_1 = CH_2CH_2OH$, $R_2 = N{=}N{-}N(C_2H_5)_2$, $R_3 = COOC_2H_5$ (*N*-hydroxyethyl-diethyltriazeno-CEP) | 0† | 4 |

* The results were expressed as mean ± S.E.M. three or four experiments in which the c.p.m. were in the range 4000–5000 for the controls (taken as 100 per cent) and 50–100 for the blank sample.

† The same analogues were not active on IMP biosynthesis.

into GAR in the controls under the conditions described below.

The incubation mixture contained in a final volume of 0.7 ml the following compounds in μmoles/ml glutamine, 14.3; ribose-5-phosphate, 57; ATP, 7; 3-phosphoglycerate, 20; $MgCl_2$, 7; inhibitors, 14, 7, 3.5, 1.4, 0.7 resp.; [1-$^{14}$C]glycine, 2 μCi; lyophilized extract, 20 mg; $K_2HPO_4$, 0.05 M, 0.10 ml. The samples were incubated at 38° for 30 min, then 0.5 ml of 20% $CCl_3COOH$ was added, the precipitates were removed by centrifugation at 5000 *g* for 15 min, and aliquots of 0.1 ml of 1 M $K_2HPO_4$ were added to each sample. The residual [1-$^{14}$C]glycine was removed by decarboxylation with ninhydrin, 1 ml of 0.2 mM (30 mg/ml) to a boiling water bath for 30 min. The aliquots were aerated with $CO_2$ for 15 min and diluted with water to 10 ml. Two millilitres of aliquots were mixed with 10 ml of toluene–Triton X-100 scintillation liquid and counted with a Nuclear-Chicago 4643 liquid scintillation counter.

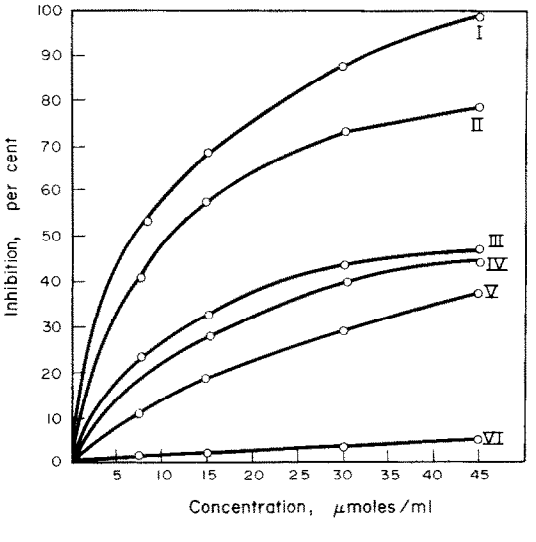
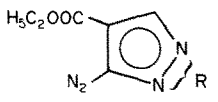



Fig. 1. Inhibition of GAR synthesis by pyrazole analogues.
I—bis-chloroethyltriazeno-CEP.
II—ACEP.
III—*N*-methyl-bis-chloroethyltriazeno-CEP.
IV—*N*-hydroxyethyl-bis-chloroethyltriazeno-CEP.
V—ACP.
VI—*N*-hydroxyethyl-ACEP.

## RESULTS AND DISCUSSION

The inhibitory effect of pyrazole derivatives on GAR synthesis at 4 μmoles/ml is given in Table 1. As shown there, the compounds tested inhibited this step to a varying extent. None of the dialkyl-(dimethyl- and diethyl-) triazeno compounds were

Table 2. Inhibitory effect of pyrazole diazonium salts on IMP biosynthesis at 4 μmoles/ml



II

| Diazonium salts | Inhibition | Number of exp |
|---|---|---|
| R = H (diazonium-CEP) | 100 | 3 |
| R = $CH_3$ (*N*-methyldiazonium-CEP) | 57 ± 6 | 3 |
| R = $CH_2CH_2OH$ (*N*-hydroxyethyl-diazonium-CEP) | 92 ± 2 | 3 |

active, and they did not inhibit inosinic acid biosynthesis. The other derivatives ACEP, ACP and all bis-chloroethyltriazeno compounds inhibited GAR synthesis.

The dose-dependence of the inhibitory action is shown in Fig. 1. As seen from the results given in this paper and in the previous one [1] the inhibitory effect of nonalkylating analogues (ACEP, ACP) and bis-chloroethyltriazenopyrazoles on GAR synthesis correlated with their effect on IMP biosynthesis. The data showed that they inhibited stronger (77–91 per cent) IMP [1] than GAR synthesis (10–40 per cent). We suggested that because of the structural resemblance of the analogues with several precursors in this metabolic pathway as AICR (5′-phosphoribosyl-5-amino-4-imidazolecarboxylate, aminoimidazolecarboxylateribotide) and AICAR (5′-phosphoribosyl-5-amino-4-imidazolecarboxamide, aminoimidazolecarboxamideribotide) they could interfere with some other enzymes taking part in purine biosynthesis *de novo* after GAR. This could explain their stronger inhibitory effect on IMP biosynthesis.

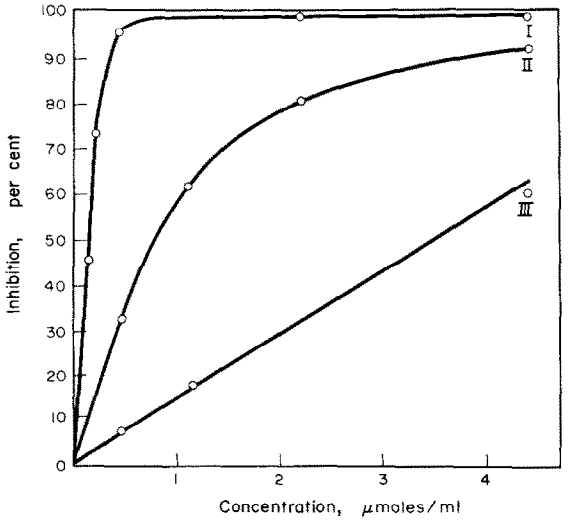



Fig. 2. Inhibition of IMP biosynthesis by pyrazole diazonium salts.
I—diazonium-CEP.
II—*N*-hydroxyethyl-diazonium-CEP.
III—*N*-methyl-diazonium-CEP.

In order to clarify the mechanism of inhibitory action of triazenopyrazoles, we tested the corresponding diazonium salts. The inhibitory effect of the salts on inosinic acid biosynthesis is given in Table 2 and the dose-dependence in Fig. 2. The data obtained showed that the diazonium salts inhibited at 4 μmoles/ml (55–100 per cent) the incorporation of [$^{14}$C]formate into IMP. This correlated well with the results obtained with bischloroethyltriazenopyrazoles (40–95 per cent) at the same concentration [1]. This allows us to suggest that in the *in vitro* system for purine nucleoside monophosphate biosynthesis *de novo*, the inhibitory action of bis-chloroethyltriazenopyrazoles is via their corresponding diazonium salts. The dialkyltriazenopyrazoles (diethyl- and dimethyl-, respectively) are not active under the conditions described. To clarify this difference in the biological activity of the two types of triazenes, we studied their rates of decomposition in aqueous solution. In Fig. 3 are given, for example, u.v. spectra of *N*-methyl-bis-chloroethyltriazeno-CEP, dimethyltriazeno-CEP and the corresponding diazonium salts in distilled water and in the presence of HCl (pH 1) after 1 and 24 hr. In neutral solutions both (bis-chloroethyl- and dimethyl)triazenopyrazoles are stable. In the presence of HCl they both slowly decompose. *N*-Methyl-bis-chloroethyltriazeno-CEP gives a breakdown product with a u.v.-spectrum, similar to the spectrum of the corresponding diazonium salts (Fig. 3A), while the spectrum of dimethyltriazeno-CEP breakdown product is different (Fig. 3B). The same results were obtained with the other triazenes. It is known that the decomposition of some triazenoimidazoles is light catalysed [11]. Since all our experiments were performed in light, we do not discuss its effect.

The difference in the inhibitory action of the two types of triazenopyrazoles is evidently not due to a difference in spontaneous breakdown of the compounds under the conditions described. We suggest that they follow different mechanisms of enzyme degradation, which is going to be the subject of our further studies.

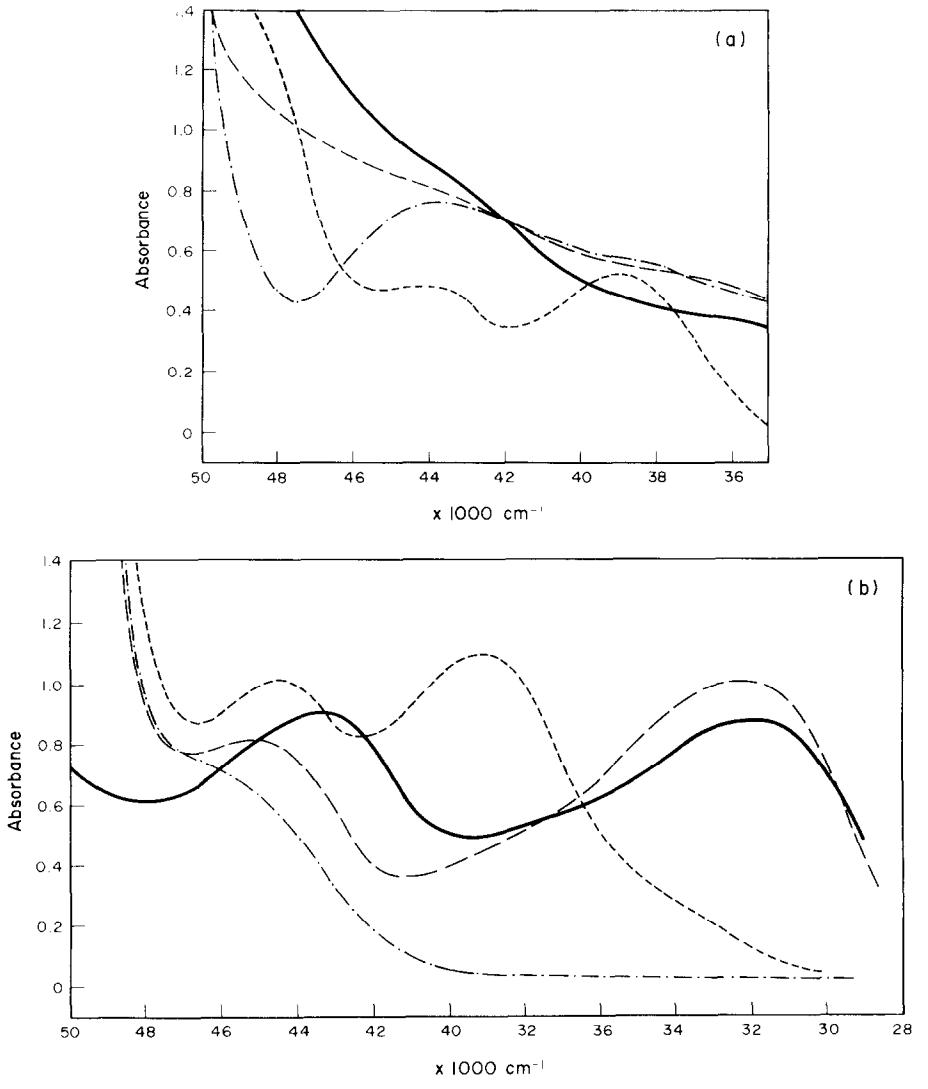



Fig. 3. u.v. Spectra of triazenopyrazoles at different pH.
A—*N*-methyl-bis-chloroethyltriazeno-CEP.
—— in dist. $H_2O$; after 24 hr at room temperature the spectrum was unchanged.
—— in the presence of HCl (pH 1).
·—·— in the presence of HCl after 24 hr.
---- *N*-methyldiazonium-CEP.
B—dimethyltriazeno-CEP.
—— in dist. $H_2O$; after 24 hr at room temperature the spectrum was unchanged.
—— in the presence of HCl (pH 1).
·—·—· in the presence of HCl after 24 hr.
---- diazonium-CEP.

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