pyridinecarboxaldehyde, and 2.5 g (20 mmoles) of DBN[26] in 75 ml of anhyd DMF was allowed to stand at ambient temp for 24 hr, then poured into 125 ml of H_2O. The resulting soln was decanted from the oil which formed. The oil was dissolved in EtOH, and the soln was clarified with charcoal, then treated by method D without further purification.

4-[3-(3,4-Dichlorophenoxy)-1-propenyl]pyridine (method C) was prepd by the above procedure from **54** and 3,4-dichlorophenoxyacetaldehyde[23,24] and treated by method D without further purification.

4-[3-(3,4-Dichlorophenoxy)propyl]pyridine (47) (Method D).—A soln of 0.50 g (1.8 mmoles) of 4-[3-(3,4-dichlorophenoxy)-1-propenyl]pyridine in 100 ml of EtOH was shaken with 2–3 atm of H_2 and 50 mg of 10% Pd/C until the theor amt was used. The soln was treated with charcoal, filtered, and evapd to give 0.45 g (21% from **54**) of an oil which was dissolved in Et_2O and treated with HCl gas. The resulting HCl salt was recrystd from Me_2CO–Et_2O; yield, 0.40 g, mp 164–166°. *Anal.* ($C_{14}H_{13}Cl_2NO \cdot HCl$) C, H, N.

3-(3,4-Dichlorophenoxy)-1,2-propanediol (57).—A soln of 16.3 g (0.10 mole) of 3,4-dichlorophenol and 35.0 g (0.32 mole) of 3-chloro-1,2-propanediol in 70 ml of DMF was heated at 70° with 13.8 g (0.10 mole) of K_2CO_3 for 3 days, then poured into H_2O. The crude product was collected on a filter, washed with H_2O, triturated in 2 *M* NaOH, then washed with H_2O, and finally recrystd from H_2O; yield, 13.7 g (55%) white solid, mp 105–107°. *Anal.* ($C_9H_{10}Cl_2O_3$) C, H, Cl.

3,4-Dichlorocinnamyltriphenylphosphonium Chloride (56).—A soln of 23.0 g (10.6 mmoles) of 3,4-dichlorocinnamic acid in 100 ml of $SOCl_2$ was refluxed for 20 min and then evapd *in vacuo* to a solid which was suspended in 100 ml of dioxane and added over 30 min to a stirred suspension of 25.0 g (65.7 mmoles) of $NaBH_4$ in 250 ml of dioxane. The soln was stirred at room temp for 30 min then cooled to and maintained at 20° while 60 ml of ice water and then 125 ml of 8 *M* HCl were slowly added over 1 hr. The contents of the flask were poured into 800 ml of H_2O and twice extd with $CHCl_3$. The combined org layers were washed with three 400-ml portions of satd aq $NaHCO_3$, two 400-ml portions of H_2O, dried (Na_2SO_4), and evapd to an orange liquid. This alcohol was treated with 100 ml of $SOCl_2$ as above and then evapd to a dark liquid (19.8 g, 89.5 mmoles) which was dissolved in 150 ml of PhMe contg 24.0 g (90 mmoles) of PPh_3. The resulting soln was heated overnight on a steam bath, cooled, and filtered. The collected product was recrystd from PhH–MeOH; yield, 25.0 g (57%) of white amorphous solid, mp 144–146°. *Anal.* ($C_{27}H_{22}Cl_3P \cdot 0.5H_2O$) C, H.

(26) 1,5-Diazabicyclo[4.3.0]non-5-ene; see H. Oediger, H. Kabbe, F. Moller, and E. Eiter, *Chem. Ber.*, **99**, 2012 (1966).

Irreversible Enzyme Inhibitors. 184.[1,2] An Affinity Column for Purification of Rat Liver Guanine Deaminase and Xanthine Oxidase

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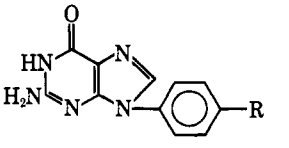
An affinity column for guanine deaminase and xanthine oxidase was prepared by coupling 9-(*p*-aminoethoxyphenyl)guanine, an inhibitor of both enzymes, to BrCN-activated Sepharose-4B. Xanthine oxidase from a 0–45% $(NH_4)_2SO_4$ fraction of rat liver was purified 230-fold with 90% recovery by absorption on the affinity column and elution with 1 m*M* substrate, hypoxanthine. The xanthine oxidase could also be eluted with an inhibitor, 2-benzylthiohypoxanthine; the latter was readily removed by 2-octanol extraction, and the recovery of purified enzyme was 68%. Guanine deaminase from a 45–90% $(NH_4)_2SO_4$ fraction of rat liver was purified 200-fold with 87% recovery by elution with 0.5 m*M* guanine.

The design, synthesis, and enzymic evaluation of active-site-directed irreversible inhibitors[3] of guanine deaminase[4] and xanthine oxidase[5–10] have been previously reported from this laboratory. 9-Phenylguanine (**1**) was found to be a good reversible inhibitor of guanine deaminase, being complexed slightly better than the substrate;[11] it was then established that the 28-fold binding increment by the Ph group compared to Me was due to hydrophobic bonding to the enzyme.[12] The nature and dimensions of the hydrophobic region of the enzyme were then mapped;[13] then an appropriate leaving group with the proper dimensions for formation of a covalent bond with a polar group on the enzyme outside the active site (exo mechanism) could be placed on an inhibitor such as **3**.[4]



1, R = H
2, R = $O(CH_2)_2NH_2$
3, R = $O(CH_2)_2NHCO$
4, R = NH_2
5, R = $O(CH_2)_2NH$—Sepharose

An affinity column for enzyme purification consists of a solid polymeric support to which is attached by covalent linkage an inhibitor relatively specific for the enzyme to be purified.[14] The solid support must be attached to a position on the inhibitor that does not interfere with complex formation with the enzyme. Such a position on the inhibitor is not obvious until sufficient information becomes available with reversible inhibitors as to where a large group can be placed on the inhibitor without interfering with complex formation;[14] this is the same information needed to design an active-

(1) The work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper in this series see B. R. Baker and M. H. Doll, *J. Med. Chem.*, **14**, 793 (1971).

(3) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N. Y., 1967.

(4) B. R. Baker and W. F. Wood, *J. Med. Chem.*, **12**, 216 (1969).

(5) B. R. Baker and W. F. Wood, *ibid.*, **12**, 214 (1969).

(6) B. R. Baker and W. F. Wood, *ibid.*, **12**, 211 (1969).

(7) B. R. Baker and J. A. Kozma, *ibid.*, **11**, 656 (1968).

(8) B. R. Baker and J. A. Kozma, *ibid.*, **11**, 652 (1968).

(9) B. R. Baker and W. F. Wood, *ibid.*, **11**, 650 (1968).

(10) B. R. Baker and W. F. Wood, *ibid.*, **10**, 1106 (1967).

(11) B. R. Baker and D. V. Santi, *ibid.*, **10**, 62 (1967).

(12) B. R. Baker and W. F. Wood, *ibid.*, **10**, 1107 (1967).

(13) B. R. Baker and W. F. Wood, *ibid.*, **11**, 644 (1968).

(14) Ref 3, Chapter XIII.

site-directed irreversible inhibitor for an enzyme that forms a covalent linkage outside the active site.[3]

The polymeric support of choice[15,16] is Sepharose-4B, a cross-linked dextran. The Sepharose is activated by reaction of BrCN at pH 11, then treated with an aryl- or alkylamine group attached to an appropriate position of the inhibitor;[15,16] the nature of the covalent linkage between the inhibitor amino group and an OH group on Sepharose is unknown, but is probably a carbamate.[17]

Acyl derivatives of **2** (such as **3**) and **4**, as well as the meta isomer of **4**, are good reversible inhibitors of guanine deaminase.[4] Therefore BrCN-activated Sepharose was attached to **4** and its meta isomer. These column materials failed to complex guanine deaminase, presumably because the 9-phenylguanine moiety is too close to the Sepharose backbone and interferes with complex formation.[14,15] When the bridge distance between 9-phenylguanine and Sepharose was increased by insertion of an $O(CH_2)_2$ group on the inhibitor (**2**), the resultant column material (**5**) readily absorbed the crude enzyme; the latter could then be easily eluted with the substrate, guanine, with high purification.

For reasons similar to those for guanine deaminase, **3** is a good reversible inhibitor of xanthine oxidase.[5] Therefore the Sepharose affinity column (**5**) was also used for purification of xanthine oxidase from rat liver.

When rat liver extract was fractionated by 0–45% and 45–90% saturated $(NH_4)_2SO_4$, all the xanthine oxidase was in the first fraction and all the guanine deaminase was in the second fraction. The fractions were reconstituted in 0.05 *M* Tris buffer (pH 7.4) at 1 ml/g of liver. Although the capacity of the Sepharose–**5** column was not determined, all the guanine deaminase in 3 g of liver was readily retained on 1 ml of column material at 0°. The enzyme could not be eluted with 0.1 *M* HOAc[15,16] since it was immediately denatured by this reagent when a solution of the enzyme was checked; however, elution at 0° with 0.5 m*M* guanine, the substrate, was smooth and sharp, all the enzyme appearing in the first two 2-ml fractions. The recovery of enzyme was 87% and the purification was 200-fold; the total time for absorption and elution was about 2–3 hr. The guanine is converted to xanthine in this operation; since xanthine is not an inhibitor of guanine deaminase,[18] this conversion does not interfere with determination of enzyme activity. However, xanthine can interfere with protein determination[19] by the uv method and was therefore removed from an aliquot by dialysis before protein determination.

Guanine deaminase could be recovered in 70% yield when the nonabsorbed protein from a Sepharose column for dihydrofolate reductase[17] was passed through the Sepharose–**5** column. This supports the contention that this mild, rapid, affinity column method could be used for isolation of a series of enzymes from the same extract if each column is sufficiently specific for the desired enzyme.

Partially purified, commercial xanthine oxidase from bovine milk was readily absorbed on the affinity column (**5**). Elution with 1 m*M* hypoxanthine, a substrate, gave 88% recovery of enzyme in the first 7 ml of eluate.

Xanthine oxidase in the 0–45% $(NH_4)_2SO_4$ fraction from rat liver could not be detected by spectrophotometric assay at 290 mμ of hypoxanthine + O_2 → uric acid. However, this enzyme could be detected by the more sensitive dichlorophenolindophenol assay at 600 mμ with hypoxanthine as the substrate; there was a large base line in the absence of hypoxanthine due to extraneous reduction of the indophenol. When the 0–45% $(NH_4)_2SO_4$ fraction from rat liver was passed through a column of **5**, all of the xanthine oxidase was retained and all of the activity causing extraneous reduction of the indophenol passed through the column. The xanthine oxidase was eluted with 1 m*M* hypoxanthine; the recovery of enzyme was 90% and the purification was 230-fold. The xanthine oxidase could also be eluted with the reversible inhibitor, 2-benzylthiohypoxanthine;[20] the latter was readily removed from the eluate by extraction with 2-octanol with no destruction of the enzyme, the recovery being about 70%.

A series of active-site-directed irreversible inhibitors of dihydrofolate reductase that could inactivate the enzyme from Walker 256 rat tumor with little inactivation of the crude rat liver enzyme, or could inactivate the enzyme from L1210 mouse leukemia with little inactivation of the crude mouse liver enzyme, were reinvestigated with liver enzyme purified with an affinity column for dihydrofolate reductase.[17] All the compounds could then inactivate the purified dihydrofolate reductase from liver as well as the enzyme from the tumor; the failure to inactivate the enzyme from liver was attributed to the rapid hydrolysis of the SO_2F moiety to SO_3H by a "sulfonyl fluoridase" present in crude liver extract,[21] but absent in dihydrofolate reductase purified by an affinity column.[17] Later 6 irreversible inhibitors of L1210 dihydrofolate reductase were found that gave low inactivation of the purified mouse liver enzyme;[22] this latter specificity was attributed to differences in the structure of the enzyme from L1210 and mouse liver and the inability of the liver sulfonyl fluoridase to hydrolyze these compounds.[22]

In view of the mode of specificity of the irreversible inhibitors of dihydrofolate reductase, the irreversible inhibitors of guanine deaminase, previously reported to show more inactivation of the Walker 256 enzyme than rat liver enzyme, were reinvestigated with rat liver guanine deaminase purified by the affinity column, **5**. With 4 out of 5 compounds (Table I), the amount of inactivation was essentially the same with either crude or purified guanine deaminase from rat liver; thus this specificity is more probably due to differences in enzyme structure of the guanine deaminase from Walker 256 and rat liver.

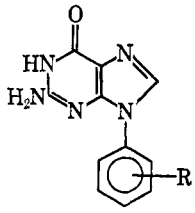
Experimental Section

Solutions.—Buffer was 0.05 *M* Tris, pH 7.4. Guanine (0.5 m*M*) was prepared by diln of 1 m*M* guanine in 0.1 *M* NaOH with an equal vol of buffer giving a soln of pH 9. Hypoxanthine (1 m*M*) was prepd by diln of 50 m*M* hypoxanthine in 0.1 *M* NaOH with buffer. Rat liver was extd, then fractionated into 0–45%

(15) P. Cuatrecasas, M. Wilcheck, and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U. S.*, **61**, 636 (1968).
(16) P. Cuatrecasas, *J. Biol. Chem.*, **245**, 3059 (1970).
(17) B. R. Baker and N. M. J. Vermeulen, *J. Med. Chem.*, **13**, 1143 (1970).
(18) B. R. Baker, *ibid.*, **10**, 59 (1967).
(19) O. H. Lowry, C. A. Smith, and D. L. Cohen, *J. Biol. Chem.*, **146**, 519 (1942).
(20) B. R. Baker and J. L. Hendrickson, *J. Pharm. Sci.*, **56**, 955 (1967).
(21) A. J. Ryan, N. M. J. Vermeulen, and B. R. Baker, *J. Med. Chem.*, **13**, 1140 (1970).
(22) B. R. Baker and N. M. J. Vermeulen, *ibid.*, **13**, 1154 (1970).

TABLE I

IRREVERSIBLE INHIBITION OF GUANINE DEAMINASE BY



No.[a]	R	% inactivation[b] Walker 256.[a]	Crude rat liver[a]	Purified rat liver[c]
20	*m*-NHCOCH$_2$C$_6$H$_4$SO$_2$F-*p*	54	36	14
21	*m*-NHCO(CH$_2$)$_2$C$_6$H$_4$SO$_2$F-*p*	68	26	15
22	*m*-NHCO(CH$_2$)$_4$C$_6$H$_4$SO$_2$F-*p*	62	33	19
31	*p*-O(CH$_2$)$_2$NHCOC$_6$H$_4$SO$_2$F-*p*	46	0	28
33	*p*-O(CH$_2$)$_2$NHCONHC$_6$H$_4$SO$_2$F-*p*	43	28	26

[a] Number and data except for the last column are taken from ref 4. [b] Enzyme incubated with 12 μM inhibitor at 37° for 60 min, then excess inhibitor removed by 2-octanol extn, and the remaining enzyme assayed as previously described.[4] [c] The technical assistance of Janet Wood with these assays is acknowledged.

and 45–90% satn of $(NH_4)_2SO_4$ as previously described;[23] the protein was redissolved at 1 ml/g of original rat liver, then stored in aliquots at −15° where enzyme activity was stable for months.

Enzyme Assays.—Guanine deaminase was assayed spectrophotometrically with 13.3 μM guanine in buffer as previously described.[18] Xanthine oxidase could not be detected in exts from Walker 256 and rat liver when assayed at 290 mμ to follow the air oxidn of hypoxanthine to uric acid;[20] however, the enzyme in these exts could be assayed by the more sensitive reduction of 2,6-dichlorophenolindophenol (DCPI) as hypoxanthine was oxidized. The assay was performed[6] in a 1-ml glass cuvette contg 0.75 ml of buffer, 50 μl of 320 μM hypoxanthine (cuvette concn = 16 μM) in buffer, 100 μl of DCPI (0.1 mg/ml), and 100 μl of enzyme in buffer by following the rate of decrease of OD at 600 mμ on a Gilford recording spectrophotometer.

Sepharose-4B Affinity Column (5).—To a stirred mixt of 4 ml of wet Sepharose-4B in 4 ml of H_2O was added a soln of 0.4 g of BrCN in 4 ml of H_2O in 1 portion. The pH was kept at 11 by addn of 4 *N* NaOH, reaction being complete in 8 min. This activated Sepharose-4B was collected on a Buchner funnel and washed with ~20 ml of 0.1 *M* $NaHCO_3$ in portions.[15,16]

The activated Sepharose-4B was added immediately to a filtered soln of 100 mg of **2**[5] in 10 ml of DMF and 15 ml of DMSO. After being stirred at ambient temp for about 18 hr, the mixt was filtd, and the Sepharose-**5** was washed with DMF, then H_2O until no more uv-absorbing material at 270 mμ could be detected. This material was stored at 3° as an aq suspension until ready for use.

Purification of Guanine Deaminase by the Affinity Column (5).—A mixt of 1 ml of wet **5** and 2 ml of untreated Sepharose-4B was poured into a column of 8-mm diam, placed in an ice bath made from an inverted, bottomless polyethylene bottle, then washed with cold buffer. The 45–90% $(NH_4)_2SO_4$ fraction from rat liver was dild with 2 vol of buffer; 5 ml of this soln contg 57 mg of protein[19] and 1.29 units[24] of guanine deaminase activity was passed through the column. The column was then washed with ice-cold buffer (about 12–15 ml) until no more protein was detectable by uv.[19] The enzyme was then eluted with 0.5 mM guanine and the eluate, caught in 2-ml fractions, was kept in an ice bath; all the enzyme activity (1.13 units) appeared in the first 2 fractions. The two 2-ml fractions were combined and an aliquot was dialyzed at 3° against buffer for 30 hr in a 1-ml microdialysis apparatus (Chemical Rubber Co.). Protein detn by uv[19] showed a total of 0.28 mg in the 4 ml of eluate. The recovery of guanine deaminase activity was 87% and the purification 200-fold; the activity was stable indefinitely at −15°. The column material was washed with 20 ml of buffer and was then ready for reuse.

The nonabsorbed protein from a dihydrofolate reductase affinity column[17] could also be used for isolation and purification of guanine deaminase with the affinity column, **5**; the recovery of guanine deaminase activity was 70%.

Purification of Xanthine Oxidase by the Affinity Column (5). (A). Substrate Elution.—The 0–45% $(NH_4)_2SO_4$ fraction from rat liver was dild with 2 vol of buffer; 5 ml of this soln, which contd 121 mg of protein,[19] 16.0 units[24] of extraneous dichlorophenolindophenol reductase activity (base line with no hypoxanthine), and 18.0 units[24] of xanthine oxidase activity, was passed through the column (**5**) as described above for guanine deaminase. The column was then washed with buffer until no more protein was detectable by uv; all of the extraneous indophenol reductase passed through the column. The xanthine oxidase was then eluted with 1 mM hypoxanthine in buffer. The activity (14.4 units) appeared in the first 5.4 ml of eluate which contd 0.53 g of protein (after dialysis of an aliquot). The recovery was 90% and the purification was 230-fold. Activity was greatly diminished after 24 hr at 3°, but the activity was stable for at least several weeks at −15°.

Similarly, 13.4 units[24] of bovine milk xanthine oxidase (Sigma Chem. Co., No. X0375) could be absorbed on the affinity column (**5**), then eluted with 1 mM hypoxanthine with recovery of 11.8 units (88%).

(B). Inhibitor Elution.—Through the affinity column (**5**) was passed 3 ml of dild rat liver ext contg 15.2 units[24] of xanthine oxidase as in procedure A above. After the column was washed with 10 ml of buffer, the xanthine oxidase was eluted with an 0.2 mM soln of 2-benzylthiohypoxanthine[20] prepd by dissolving 5.16 mg of the inhibitor in 5 ml of DMSO, then diln to 100 ml with buffer. All of the activity appeared in the first three 2-ml fractions. Each fraction was washed twice with 2 ml of H_2O-satd 2-octanol to remove the inhibitor, then assayed. The 6 ml of eluate contd 10.3 units (68% recovery) of xanthine oxidase free of the extraneous indophenol reductase.

(23) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967).

(24) A unit of enzyme activity is arbitrarily defined as the amt of enzyme necessary to give an OD change of 1 unit/min under the assay conds.