brik, Konstanz, FRG), sodium nitroprusside, and isosorbide dinitrate were markedly inhibited. Inhibition was almost maximal at 1 μM Ca^{2+}. Basal guanylate cyclase activity was unaffected over the Ca^{2+} concentration range of 0.02–10 μM, irrespective of whether Mn-GTP (fig. 1) or Mg-GTP served as the enzyme substrate. In the presence of Mn-GTP, basal guanylate cyclase activity was approximately 6-fold higher than in the presence of Mg-GTP.

In order to rule out that the observed effects of free Ca^{2+} on guanylate cyclase stimulation represent a unique property of arterial guanylate cyclase, similar experiments were carried out on a 100,000 × g supernatant obtained from rat liver. In this preparation, the dependence of guanylate cyclase stimulation by dinitro-NECA and sodium nitroprusside on Ca^{2+} was very similar to that observed in the arterial supernatant (fig. 2). Again, basal guanylate cyclase activity proved to be Ca^{2+} independent, irrespective of whether Mn-GTP or Mg-GTP served as the substrate.

Thus, guanylate cyclase stimulation by the nitro-compounds dinitro-NECA, sodium nitroprusside, and isosorbide dinitrate was strongly inhibited by free Ca^{2+} concentrations well within the physiological range, i.e., the half-maximal inhibitory concentration of Ca^{2+} was about 0.5 μM. Since the basal guanylate cyclase activity proved to be completely independent of Ca^{2+}, a direct interaction of Ca^{2+} and the nitro-compounds on the guanylate cyclase molecule may be assumed. However, the hypothesis is rather speculative at present due to the lack of experimental evidence for the putative nitro- or nitroso-receptor. A possible role of the Ca^{2+}-binding protein, calmodulin, is obviously ruled out since the calmodulin-antagonistic compound trifluperazine[12] (0.1 mM) was without any influence on the observed Ca^{2+} inhibition (data not shown). Nevertheless, due to its steep concentration-response curve, minor changes in the level of free Ca^{2+} must have marked effects on guanylate cyclase activation by nitro-compounds. Hence, their guanylate cyclase stimulatory action appears to be sensitively modulated in vivo by intracellular variations of the free Ca^{2+} concentrations.

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2 Mittal, C.K., and Murad, F., Cyclic Nucleotides, Handbook exp. Pharmac. vol. 58/I, p. 225. Eds J.W. Kebabian and J.A. Nathanson. Springer, Berlin/Heidelberg/New York 1982.
3 Ignarro, L.J., Lippton, H., Edwards, J.C., Baricos, W.H., Hyman, A.L., Kadowitz, P.J., and Gruetter, C.A., J. Pharmac. exp. Ther. *218* (1981) 739.
4 Gruetter, C.A., Barry, B.K., McNamara, D.B., Gruetter, D.Y., Kadowitz, P.J., and Ignarro, L.J., J. cyclic Nucleot. Res. *5* (1979) 211.
5 Kimura, H., and Murad, F., J. biol. Chem. *249* (1974) 6910.
6 Kimura, H., Mittal, C.K., and Murad, F., J. biol. Chem. *251* (1976) 7769.
7 Chrisman, T.D., Garbers, D.L., Parks, M.A., and Hardman, J.G., J. biol. Chem. *250* (1975) 374.
8 Criss, W.E., Murad, F., and Kimura, H., J. cyclic Nucleot. Res. *2* (1976) 11.
9 Birnbaumer, L., Torres, H.N., Flawia, M.M., and Fricke, R.F., Analyt. Biochem. *93* (1979) 124.
10 Piascik, M.T., Wisler, P.T., Johnson, C.L., and Potter, J.D., J. biol. Chem. *255* (1980) 4176.
11 Kukovetz, W.R., Holzmann, S., Wurm, A., and Pöch, G., Naunyn-Schmiedeberg's Arch. Pharmac. *310* (1979) 129.
12 Potter, J.D., Piascik, M.T., Wisler, P.L., Robertson, S.R., and Johnson, C.L., Ann. N.Y. Acad. Sci. *356* (1980) 220.

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Inhibition of brain enolases by acrylamide and its related compounds in vitro, and the structure-activity relationship

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Summary. Acrylamide and its related compounds inhibited brain enolases in vitro independently of their neurotoxicity. The inhibitory potency was a function of the binding constants of the compounds for phenylalanine. The binding constant for tryptophan was higher in neurotoxic compounds than in non-neurotoxic ones.
Key words. Rat brain; Enolase; acrylamide; acrylamide derivatives; neurotoxicity; structure-activity relationship.

It is well-known that acrylamide produces peripheral neuropathy in experimental animals and human beings[1,2]. Neurotoxicity has also been reported for some analogues, including N-hydroxymethylacrylamide[3-5], methacrylamide[4-6], N-isopropylacrylamide[4,5], and N-methylacrylamide[4,5] in mice and/or rats. Studies on the etiology of acrylamide neuropathy have suggested that inhibition of neuronal glycolytic enzymes such as enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by acrylamide might produce toxic distal axonopathies[7-12]. A recent study[13] in our laboratory, however, showed that both mouse brain total enolase and GAPDH were inhibited in vitro not only by neurotoxic acrylamide derivatives but also by non-neurotoxic ones. The present study was undertaken to examine further the inhibitory potency of the compounds for enolase isozymes in rat brain. The structure-activity relationship of the test compounds was also investigated in connection with their physico-chemical properties and the inhibitory potency on enolase.

Methods. Male Wistar rats (180–200 g) were used throughout the experiment. Three forms of brain enolase, a neuron-specific form ($\gamma\gamma$), a hybrid form ($\alpha\gamma$), and a non-neuronal form ($\alpha\alpha$), were separated by DEAE-cellulose column chromatography according to the method of Francis et al.[14]. Rats were killed by decapitation and brains were removed and homogenized (20% w/v) in cold 10 mM potassium phosphate buffer containing 1 mM magnesium sulfate, pH 7.4, using glass homogenizers fitted with Teflon pestles. Homogenates were centrifuged at 10,000 g for 20 min. The resulting supernatants were chromatographed using DEAE-cellulose (Whatman DE 32) column (25 × 2.0 cm). The column was washed successively with 3 portions of 10 mM phosphate buffer (1 mM $MgSO_4$, pH 7.4) containing 40, 130 and 240 mM KCl to elute each enolase. The

protein was determined by the method of Lowry et al.[15], using bovine serum albumin as the standard.

Enolase activity was measured by a spectrophotometric assay[16]. Each I_{50} (inhibitory constant, based on concentrations of test compounds showing 50% inhibition of the enolase activity) was estimated by plotting the relation between the enzyme activity and the concentration used. The binding constant (K) of the test compounds with either phenylalanine or tryptophan was measured by determining the quenching of fluorescence of the amino acids by the test compounds. To cuvettes containing phenylalanine (0.40 mM) or tryptophan (0.49 mM) in 0.1 M potassium phosphate buffer (pH 7.4) the test compound was added to make a final volume of 3.0 ml. Wave lengths (nm) of excitation and emission used were 265 and 287 for phenylalanine, and 285 and 355 for tryptophan, respectively. Binding constants were calculated from the reciprocal plots according to Woronick[17]. Determination of the bimolecular rate constants for the reaction of test compounds with reduced glutathione was carried out by the method of Hashimoto and Aldridge[18]. n-Octanol-water partition coefficients (P) for the compounds were determined as described previously[19]. The structure-inhibitory potency relationship was analysed by Hansch's model[20] using log (binding constant), log (rate constant) or log (partition coefficient) as structural factors of the molecules.

Results. All experimental data are summarized in the table. There were no significant differences in the I_{50} values for all test compounds among 3 enolase isozymes. No definite relation to the inhibitory potency was found on comparison of neurotoxic and non-neurotoxic compounds. Of the structural factors, the binding constant (K) for phenylalanine was found to correlate highly with log $(1/I_{50})$ for 3 enolase isozymes, and the regression equations were expressed as follows:

$\log (1/I_{50})\gamma\gamma = -0.725(\log K)^2 - 0.286\log K + 0.143$
$(n = 10, r = 0.976, s = 0.091)$
$\log (1/I_{50})\alpha\gamma = -0.707(\log K)^2 - 0.290\log K + 0.140$
$(n = 10, r = 0.975, s = 0.093)$
$\log (1/I_{50})\alpha\alpha = -0.699(\log K)^2 - 0.309\log K + 0.160$
$(n = 10, r = 0.968, s = 0.107)$

where n is the number of compounds, r is the correlation coefficient and s is the standard deviation. Each equation was statistically significant ($p < 0.01$). The binding constant for tryptophan, on the other hand, revealed no relation to I_{50}, but showed some connection with the neurotoxic potency of the compounds, having significantly higher values for neurotoxic compounds than in non-neurotoxic ones. Other structural factors, i.e. rate constant for reduced glutathione and log P, were unlikely to have any correlation with I_{50}.

Discussion. Enolase in nervous tissues has been found to be inhibited by acrylamide in vivo and in vitro[10-13], and the inhibition is postulated to be involved in the meachnism of the neuropathy due to the compound[10-12]. In the present study, however, non-neurotoxic as well as neurotoxic compounds were found to inhibit brain enolases in vitro, and no correlation between the inhibitory potency and neurotoxicity was seen. On the other hand, the binding constant for tryptophan seemed to be correlated with the neurotoxicity of the compounds, although the underlying mechanism is as yet unknown. No other structural factors were found to be related to the potency of enolase inhibition. The study on the structure-activity relationship has revealed that the variation in potency of the test compounds for enolase inhibition is largely dependent on log K, the binding constant for phenylalanine, and this might indicate the importance of their reactivies with phenylalanine in the inhibition of brain enolases.

I_{50}, binding constant, rate constant, partition coefficient and neurotoxicity for acrylamide and its related compounds

Compound	Structure	I_{50} (mM) for rat brain enolases		
		$\gamma\gamma$	$\alpha\gamma$	$\alpha\alpha$
Acrylamide	$CH_2{=}CHCONH_2$	4.9	4.9	4.7
N-Hydroxymethylacrylamide	$CH_2{=}CHCONHCH_2OH$	1.8	1.8	1.9
Methacrylamide	$CH_2{=}C(CH_3)CONH_2$	6.7	6.5	6.2
N-Isopropylacrylamide	$CH_2{=}CHCONHCH(CH_3)_2$	0.9	0.9	0.9
N-Methylacrylamide	$CH_2{=}CHCONHCH_3$	2.0	2.0	2.0
N,N-Dimethylacrylamide	$CH_2{=}CHCON(CH_3)_2$	0.8	0.8	0.7
Crotonamide	$CH_3CH{=}CHCONH_2$	3.5	3.5	3.4
N,N-Diethylacrylamide	$CH_2{=}CHCON(C_2H_5)_2$	0.7	0.7	0.7
N-*tert*-Butylacrylamide	$CH_2{=}CHCONHC(CH_3)_3$	1.0	1.0	1.0
Diacetone acrylamide	$CH_2{=}CHCONHC(CH_3)_2CH_2COCH_3$	0.6	0.6	0.5

Binding constant (mM) Phenylalanine	Tryptophan	Rate constant ($M^{-1}\cdot min^{-1}$)	logP	Neurotoxicity[a]
6.10	46.0	0.910	−0.76	+
3.24	41.6	0.910	−1.02	+
10.3	36.8	0.014	−0.23	+
1.57	25.6	0.022	0.49	+
4.57	56.2	0.058	−0.36	+
0.48	2.46	0.095	−0.60	−
6.66	2.50	−[b]	−0.47	−
0.37	1.69	0.058	−2.18	−
2.02	5.76	0.014	1.10	−
1.27	6.25	0.056	0.23	−

All values are the mean of 3 determinations; [a] Cited from Hashimoto et al.[4] and Tanii et al[5]. +, neurotoxic; −, non-neurotoxic; [b] Too small to be determined.

1 Spencer, P.S., and Schaumburg, H.H., Can. J. neurol. Sci. *1* (1974) 143.
2 Spencer, P.S., and Schaumburg, H.H., Can. J. neurol. Sci. *1* (1974) 151.
3 Edwards, P.M., Biochem. Pharmac. *24* (1975) 1277.
4 Hashimoto, K., Sakamoto, J., and Tanii, H., Archs Toxic. *47* (1981) 179.
5 Tanii, H., and Hashimoto, K., Archs Toxic. *54* (1983) 203.
6 Drees, D.T., Crago, F.L., Hopper, C.R., and Smith, J.M., Toxic. appl. Pharmac. *37* (1976) 190.
7 Spencer, P.S., Sabri, M.I., Schaumburg, H.H., and Moore, C.L., Ann. Neurol. *5* (1978) 501.
8 Sabri, M.I., Ederle, K., Holdsworth, C.E., and Spencer, P.S., Neurotoxicology *1* (1979) 285.
9 Sabri, M.I., Moor, C.L., and Spencer, P.S., J. Neurochem. *32* (1979) 683.
10 Howlands, R.D., Vyas, I.L., and Lowndes, H.E., Brain Res. *190* (1980) 529.
11 Howlands, R.D., Vyas, I.L., Lowndes, H.E., and Argentieri, T.M., Brain Res. *202* (1980) 131.
12 Howlands, R.D., Toxic. appl. Pharmac. *60* (1981) 324.
13 Sakamoto, J., and Hashimoto, K., Neurotoxicology, in press (1983).
14 Francis, A., Rivett, A.J., and Roth, J.A., Brain Res. *263* (1983) 89.
15 Lowry, O.H., Rosebrough, N.J., Faar, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
16 Odelstad, L., Pahlman, S., Nilsson, K., Larsson, E., Läckgren, G., Johansson, K.E., Hjerten, S., and Grotte, G., Brain Res. *224* (1981) 69.
17 Woronick, C.L., Acta chem. scand. *17* (1963) 1789.
18 Hashimoto, K., and Aldridge, W.N., Biochem. Pharmac. *19* (1970) 2591.
19 Tanii, H., and Hashimoto, K., Toxic. Lett. *11* (1982) 125.
20 Hansch, C., and Fujita, T., J. Am. chem. Soc. *86* (1969) 1616.

0014-4754/84/090971-02$1.50 + 0.20/0