Stabilization of Mouse Brain Glutamic Decarboxylase

Attempts to purify L-glutamate 1-carboxy-lyase, EC 4.1.15 (GAD) from mouse brain, a rich source of this enzyme, repeatedly had met with failure in our hands because of the lability of the enzyme. Studies with the crude enzyme showed that the GAD of mouse brain is a sulfhydryl enzyme and requires pyridoxal phosphate (PLP) for activity[1]. It now is reported that a combination of PLP and 2-aminoethyl-isothiuronium bromide (AET) can stabilize the enzyme for periods long enough to allow partial purification of the enzyme[2].

Enzyme preparation used and assay. Mouse brains minus the brain stems were removed from mice killed by cervical dislocation. A 10% homogenate was made in ice-cold 0.25 M sucrose. A fraction was removed by centrifugation for 10 min at $900\times g$ in a refrigerated centrifuge. A particulate fraction was sedimented from the supernatant fluid by centrifugation for 15 min at $17{,}400\times g$ in the No. 30 rotor of the Spinco L-2 centrifuge. The pellet was rehomogenized in 1/10 the original volume of N_2-gassed glass-distilled water. After storage for 20 min on ice to permit osmotic rupture of particles, the suspension was centrifuged for 50 min at $10{,}000\times g$. The supernatant fluid containing the enzyme was decanted and a sufficient quantity of 0.1 M potassium phosphate buffer, pH 7.2, with or without protective substances was added to make the buffer concentration in the supernatant 1×10^{-3} M.

Enzyme activity was assayed by a procedure previously described by ROBERTS and SIMONSEN[1] that depends on the evolution of $^{14}CO_2$ from L-glutamic acid-1-^{14}C under anaerobic conditions.

Soluble preparations of GAD stored in solution at 4 °C in the presence of reduced glutathione GSH and PLP were inactivated more rapidly in the light than in the dark. B_6 vitamers and B_6 enzymes are inactivated by UV- light and by sunlight[3]. The apoenzyme of GAD, as well as the coenzyme, may be inactivated by light-induced free radicals, since large excesses of PLP present in the assay mixture failed to reactivate the enzyme. GSH and PLP, both of which are known to react with free radicals in solution, protect GAD against inactivation. Samples were stored frozen in the dark and prior to assay were diluted 5-fold with solutions of the same composition as those in which they were contained originally. Regardless of the concentration of PLP employed, all of the samples preincubated for 60 min at 37 °C showed approximately a 15% loss of activity. However, increasing the amounts of PLP decreased the extent of loss of activity during a 4-day period of storage in the cold room. With 2×10^{-4} M PLP and varying concentrations of GSH, full activity was maintained at concentrations of GSH between 1.3×10^{-3} M and 5.2×10^{-3} M during preincubation for 60 min at 37 °C. Approximately 85% of the original activity was maintained during storage for 4 days at 4 °C in the dark at concentrations of GSH of 5.2×10^{-3} M and above.

2-Aminoethylisothiouronium bromide (AET), a radioprotective substance shown by KHYM, SHAPIRA and DOHERTY[4] to exist in neutral solution as 2-mercaptoethylguanidine, protected GAD somewhat better than isomolar amounts of GSH (Table I). Portions of a typical enzyme preparation were dissolved in 0.1 M potassium phosphate buffer, pH 7.2, which had PLP (1×10^{-4} M) and either AET (1×10^{-3} M) or GSH (1×10^{-3} M). Aliquots kept on ice in the dark for 18 h served as controls. Samples were irradiated at room temperature in test tubes for periods up to 18 h by an ultraviolet lamp placed 6 inches above the surface of the solutions and then were stored unfrozen on ice with the control samples until assay. Activity was measured after 5-fold dilution with 0.1 M phosphate buffer, pH 6.5, containing PLP (1×10^{-4} M) and either AET (1×10^{-3} M) or GSH (1×10^{-3} M). The samples assayed in AET-containing substrate mixture gave higher activities than the comparable ones assayed in GSH in the unirradiated controls and, with one exception, at various times after the beginning of the irradiation; and those preincubated in AET had higher activities than those preincubated in GSH at the corresponding time intervals, whether the dilution prior to assay was made with buffer containing AET or GSH.

[1] E. ROBERTS and D. G. SIMONSEN, Biochem. Pharm. *12*, 113 (1963).
[2] J. P. SUSZ, B. HABER and E. ROBERTS, Biochemistry *5*, 2870 (1966).
[3] P. SCHLENK, A. FISHER and E. E. SNELL, Proc. Soc. exp. Biol. Med. *67*, 183 (1946).
[4] J. X. KHYM, R. SHAPIRA and D. G. DOHERTY, J. Am. chem. Soc. *79*, 5663 (1957).

Table I. Comparison of protective effects of AET and GSH on mouse brain GAD activity

Time of UV-irradiation[a] (h)	$^{14}CO_2$ (cpm/assay)				
	Preincubation	AET[b]	AET[c]	GSH[c]	GSH[c]
	Assay	AET	GSH	AET	GSH
0		5970	5526	5050	4740
1		4895	4635	5172	4160
2		4820	4155	3750	3305
4		4518	3765	3216	2715
5		3715	3345	2725	2185
18		1596	1280	294	140

[a] Irradiation was carried out with a Model R-51 Mineralight, Ultra-Violet Products, South Pasadena, California. [b] The final concentrations of AET or GSH during preincubation and at time of assay were $1\times10^{-3}M$. [c] The preincubations were carried out in concentrations of $1\times10^{-3}M$ AET or GSH. Prior to assay the samples were diluted 1:5 with buffer containing GSH or AET, respectively, so that the concentrations during assay were $2\times10^{-4}M$ AET and $1\times10^{-3}M$ GSH or $2\times10^{-4}M$ GSH and $1\times10^{-3}M$ AET.

Table II. Protective effects on GAD activity of 2-aminoethylisothiouronium bromide (AET) and dithioerythritol (DTE)[a]

Treatment of enzyme	$^{14}CO_2$ (cpm/assay)			
	AET		DTE	
	$4\times10^{-4}M$	$8\times10^{-4}M$	$4\times10^{-4}M$	$8\times10^{-4}M$
None	1415	1348	1197	1172
4°C, UV, 2 h	1377	1533	1209	1160
4°C, UV, 4 h	1224	1286	1159	1057
4°C, UV, 6 h	1175	1279	1033	983
4°C, UV, 12 h	833	974	719	736
4°C, UV, 22 h	579	843	435	523

[a] The enzyme preparations were dissolved in 0.1 M phosphate buffer, pH 6.5, which contained $2\times10^{-4}M$ PLP with other additions, as indicated. Samples were removed at the required times, frozen, and kept frozen in the dark. Assays were performed on thawed samples which were diluted 5-fold with solutions of the same composition as those in which the samples originally were dissolved.

In Table II is shown a comparison of the protective effect of AET with that of dithioerythritol (DTE), another substance shown by CLELAND[5] to be a sulfhydryl protective agent. Higher activities were observed with AET at various times of UV-irradiation at 4 °C. Other experiments showed that storage of the enzyme in an opaque container up to 24 h at 4 °C in the presence of 2×10^{-4} *M* PLP did not result in any loss of activity and that AET did not increase the activity above the control level. However, an 87% loss of activity occurred upon standing for 24 h at room temperature with PLP alone, and only approximately 25% less was found in the presence of 8×10^{-4} *M* AET. Likewise, UV-irradiation for 24 h at 4 °C produced an 81% loss of activity in the absence of an SH compound and only a 41% loss in 8×10^{-4} *M* AET. UV-irradiation of buffer solutions containing PLP, AET or PLP and AET prior to addition of enzyme had no inhibitory effect on enzyme activity.

Putting PLP ($1\text{--}2 \times 10^{-4}$ *M*) and AET (1×10^{-3} *M*) in all solutions and use of opaque containers gave good protection during various steps necessary to begin purification of GAD. The above combination also has been found by STONER[6] to be highly effective in preventing loss of activity of a soluble preparation containing the GABA transaminase-succinic semialdehyde dehydrogenase systems from *Pseudomonas fluorescens* during extraction, lyophilization, and prolonged storage.

From spectral data, to be published elsewhere, it appears that AET itself may form a cyclic derivative with PLP or may be a nascent source of mercaptoethylamine, which forms a thiazolidine derivative with the aldehyde group of PLP[7]. From inhibition data, a thiazolidine linkage has been postulated by ROBERTS, WEIN, and SIMONSEN[8] to exist in GAD. The sensitivity to destruction by free radicals of the added thiazolidine complex would be expected to be of the same order as that of the active site on the enzyme; and, therefore, the probability of the active site on a particular enzyme molecule being destroyed by a given concentration of free radicals might be less in the presence of a given concentration of the complex than in the presence of similar concentrations of glutathione and pyridoxal phosphate. Perhaps the above type of mechanism could partly help to explain the superior in vivo radioprotective efficacy of AET and aminothiols, in general, when compared with sulfhydryl compounds not containing amino groups[9].

Résumé. Le GAD du cerveau de souris, une enzyme sensible au SH, peut être purifié en présence de 2-aminoéthyl-isothiouronium-bromide (AET), une substance radioprotective. AET, qui existe en solution neutre, comme 2-mercaptoethyl guanidine, protège les enzymes qui contiennent le SH bien mieux que les quantités isomolares du GSH ou du dithioérythrétol (Cleland's reagent). En outre, il est possible que l'AET soit une source naissante de mercaptoéthylamine qui produit un dérivatif de thiazolidine avec le groupe aldéhyde de pyridoxal phosphate qui opère indépendamment comme galeyeur. Ce mécanisme explique la qualité radioprotective supérieure de l'AET et des aminothiols in vivo en comparaison des substances qui ne contiennent pas d'amino.

E. ROBERTS, MARIA SZABO[10] and B. HABER[11]

Division of Neurosciences, City of Hope Medical Center, Duarte (California, USA), 3 January 1972.

[5] W. W. CLELAND, Biochemistry *3*, 480 (1963).
[6] G. A. STONER, personal communication (1966).
[7] M. V. BUELL and R. E. HANSEN, J. Am. chem. Soc. *82*, 6042 (1960).
[8] E. ROBERTS, J. WEIN and D. G. SIMONSEN, in *Vitamins and Hormones* (Eds. L. HARRIS and P. WOOL; Academic Press, New York 1964), vol. 22, p. 503.
[9] Supported by the Institute for Advanced Learning in the Medical Sciences, City of Hope, and by PHS grants No. NB-01615 and No. MH 19502, and by a grant from the Moody Foundation of Galveston.
[10] Present address: Institute of Medical Chemistry, Budapest (Hungary).
[11] Present address: Division of Comparative Neurobiology, Marine Biomedical Institute, Galveston (Texas 77550, USA); (Adress for reprint requests).

Nachweis der Bisulfit-Addukte (α-Oxysulfonsäuren) von Carbonylverbindungen in den mit SO_2 behandelten Erbsenkeimlingen

Schwefeldioxid ist ein chemisch sehr reaktives Gas, das im Pflanzenorganismus viele metabolische Vorgänge beeinflussen kann. Es ist bekannt, dass SO_2 die Intensität der Photosynthese erniedrigt[1–3], unter anderem als Folge der Hydrolyse des Chlorophylls zu Phaeophytin[4,5]. Weiter beeinflusst SO_2 den Stoffwechsel der Disulfide durch deren Überführung in S-Sulfoderivate und Sulfide[6,7]. Auch die Beeinflussung des Saccharidstoffwechsels durch SO_2 ist mehrfach bestätigt worden[8–12]. Vor allem wurde eine Erniedrigung des Saccharose-Gehalts beobachtet[9–12]. In unserer früheren Mitteilung[12] wurde über einen gleichzeitigen Anstieg des Alanin- sowie des Methioninsulfoxid-Gehalts und den Rückgang des Glutaminsäure-Gehalts berichtet.

SO_2 reagiert im wässrigen Milieu in vitro mit Carbonylverbindungen (Aldehyde, Ketone, Ketosäuren) unter Bildung von Bisulfit-Addukten (α-Oxysulfonsäuren). Diese Verbindungen hemmen die enzymatische Oxidation des Glykolats und Laktats[13,14] und Glyoxal-Bisulfit hemmt die CO_2-Fixierung[15]. Die Entstehung dieser metabolisch aktiven Bisulfit-Addukte im Pflanzengewebe kann verschiedene Störungen des Stoffwechsels zur Folge haben und wir haben die eventuelle Bildung dieser Stoffe in vivo, und zwar in Keimlingen, die mit SO_2 begast wurden, näher verfolgt.

[1] M. D. THOMAS, Pl. Physiol. *10*, 291 (1935).
[2] G. R. HILL, Pl. Physiol. *12*, 309 (1937).
[3] H. W. de KONING und Z. JEGIER, Atmos. Envir. *2*, 321 (1968).
[4] W. DORRIES, Z. PflKrankh., PflPath, PflSchutz *42*, 257 (1932).
[5] J. MÜLLER, Naturwissenschaften *44*, 453 (1957).
[6] S. G. WALEY, Biochem. J. *71*, 132 (1959).
[7] L. BALABAJEVA und I. GANČEV, Cslka hyg. *12*, 376 (1967).
[8] P. R. MILLER, F. W. COBB JR. und E. ZAVARIU, Hilgardia *39*, 135 (1968).
[9] S. BOERTITZ, Biol. Zbl. *83*, 501 (1964).
[10] I. SPÁLENÝ, F. GODIN und B. MAŘAN, 5th Int. Congr. Biochem., Moscow 1961, Abstr. Commun. 333.
[11] S. BOERTITZ, Arch. Forstw. *18*, 123 (1969).
[12] J. KOŠTÍŘ, I. MACHÁČKOVÁ, V. JIRÁČEK und E. BUCHAR, Experientia *26*, 604 (1970).
[13] I. ZELITCH, J. biol. Chem. *224*, 251 (1957).
[14] I. ZELITCH, Fedn. Proc. *24*, 868 (1965).
[15] K. ASADA und Z. KASAI, Mem. Res. Inst. Food Sci. Kyoto Univ. *23*, 66 (1961).