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SHORT COMMUNICATIONS

Effects of antioxidants on glutamate decarboxylase activity

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The sensitivity of glutamate decarboxylase (EC 4.1.1.15, GAD) to inhibition by oxygen has been recognised for some time [1], and published procedures for the assay of GAD activity invariably include an antoxidant in the assay mixture and usually recommend that the assay be performed under N_2 rather than air [2]. The traditionally used antoxidants have included mercaptoethanol [1], reduced glutathione [2], dithiothreitol [3], or aminoethylisothiouronium bromide (AET) [4]. All these compounds are sulphydryl reagents which act presumably by protecting the sulphydryl groups present in the region of the active site of GAD [4].

However, many sulphydryl reagents are potent inhibitors of GAD by virtue of their ability to form stable complexes either with the holoenzyme, for example 3-mercaptopropionic acid [5] and thiomalic acid [6], or by Schiff base formation directly with pyridoxal phosphate, for example L-cysteine [7].

The inhibition of GAD by concentrations of antoxidants close to those used ostensibly to protect the enzyme from inactivation has been remarked upon previously [4, 6]. The aim of the present work has therefore been to examine the dose-response effect of such sulphydryl antoxidants on GAD activity in extracts of whole mouse brain in order to determine the optimum reagent concentration that will not produce enzyme inhibition.

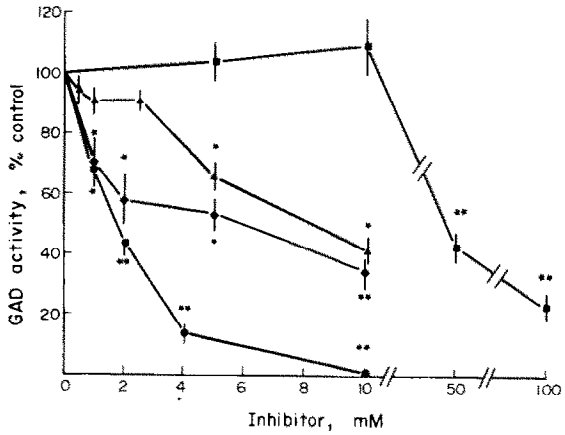

Fig. 1. Effects of varying concentrations of antoxidants on glutamate decarboxylase activity *in vitro*. Assays were performed in triplicate in the presence of L-glutamate (1 mM), pyridoxal phosphate (0.3 mM) and sodium phosphate buffer (50 mM, pH 6.8) at 37° for 30 min. Results are expressed as percentage of the control (antoxidant-free) values. Key: ■ 2-mercaptoethanol; ▲ AET; ◆ *N*-acetylcysteine; ● L-cysteinylglycine. * $P < 0.05$; ** $P < 0.005$ (two tailed student's *t* test).

Materials and Methods. The mice from which the enzyme extracts were prepared were adult LACGs of either sex bred in the department. DL-[1-^{14}C]glutamate was obtained from the Radiochemical Center, Amersham, L-cysteinylglycine from Vega-Fox Biochemicals, Tucson, Arizona, *N*-acetylcysteine from the Boehringer Corporation (London) Ltd., and all other reagents from the Sigma Chemical Co., Poole, Dorset. GAD activity was assayed in brain homogenates as described previously [6], except that no reduced glutathione was added to the preparation prior to assay. Each assay was performed at least 4 times in triplicate and enzyme activity expressed as μmoles CO_2 evolved·min^{-1}·mg^{-1} protein. Glutathione and its breakdown products were isolated by high voltage electrophoresis using methods described previously [8].

Results and discussion. The effects of 2-mercaptoethanol, AET and *N*-acetylcysteine on GAD activity are shown in Fig. 1 in which it can be seen that significant inhibition occurs with 2-mercaptoethanol at 50 mM, with AET at 5 mM and *N*-acetylcysteine at 1 mM in the presence of 1 mM L-glutamate and 0.3 mM pyridoxal phosphate. Dithiothreitol and fresh solutions of reduced glutathione (GSH) produced no inhibition at concentrations up to 10 mM. GSH solutions made up in 50 mM sodium phosphate buffer (pH 6.8) yielded measurable amounts of free glutamate and L-cysteinylglycine after storage at 4° for 2 weeks. L-Cysteinylglycine itself inhibited GAD by 30 per cent at 0.8 mM (see Fig. 1). The presence of this dipeptide in old solutions of GSH may well be responsible for the apparent inhibition of GAD by glutathione reported earlier [6]. *N*-Acetylcysteine is a relatively new sulphydryl compound marketed as an antoxidant but it is clearly too potent an inhibitor of GAD to be of use in assays of this and possibly other pyridoxal-dependent enzymes.

AET produced measurable, but not statistically significant, inhibition of GAD at concentrations (1 mM) used previously in GAD assays [4]. Mercaptoethanol and GSH appear to be the antoxidants of choice, although the former compound has an unpleasant smell and the latter should only be used freshly. Dithiothreitol, at concentrations up to 5 mM, produced no measurable inhibition and provides perhaps a better alternative.

It is interesting that GAD activity, in the relatively crude preparation used in the present study, was apparently not affected by the presence of oxygen during the assay. That is, none of the antoxidants tested produced a significant increase in enzyme activity above control values. Sulphydryl-containing antoxidants are therefore probably only of value in protecting purified preparations of GAD rather than in routine assay procedures and, even then, care should be taken not to use excessive concentrations of antoxidant.

The potentially inhibitory effects of these compounds on GAD activity are not without wider implications. Sulphydryl reagents such as thiomalic acid (in the form of its gold salt) and penicillamine are used clinically in the treatment

of rheumatoid arthritis [9], and *N*-acetylcysteine has been found useful in the treatment of paracetamol poisoning [10]. Inhibitors of GAD are well known as convulsants [3–6] and this is a possible risk if compounds such as thiomalate or *N*-acetylcysteine are able to enter the brain following systemic administration.

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Effect of the methylation of aglycone hydroxyl groups on the biological and biochemical properties of daunorubicin*

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Daunorubicin and doxorubicin are well-known anthracycline antibiotics with chemotherapeutic efficacy in the treatment of several malignant disorders. Although different mechanisms by which the anthracyclines inhibit growth of tumor cells have been proposed [1, 2], DNA is thought to be the primary target of the biological action of these anticancer agents [3, 4]. Considerable interest has been devoted by many laboratories to studies of structure–activity relationships among anthracycline derivatives [4, 5]. A positive correlation between biological activity and DNA affinity has been found in several series of daunorubicin and doxorubicin derivatives [4]. Therefore, further investigations on the structural and conformational features of drug molecules relevant to the mechanism and specificity of their binding to DNA are of interest to identify essential and nonessential elements in their structures. We have undertaken a systematic study on the influence of methylation of hydroxyl groups in the chromophore. This report describes the relation between some biochemical and biological properties of daunorubicin derivatives obtained by methylation of hydroxyl groups at the 6, 9 and 11 positions (Fig. 1).

Daunorubicin derivatives were synthesized by Farmitalia Carlo Erba, Milan, Italy (G. Cassinelli *et al.*, manuscript in preparation).

Methods for the determination of the cytotoxic effect on HeLa cells have been previously described [7]. For the determination of intracellular drug levels, HeLa cells were incubated with drug (2 μM) at 37° for 15, 30, 60 and 120 min. Saturation of intracellular drug accumulation is reached for the tested compounds within 60 min. After incubation the cells were harvested and kept frozen at −20°. The cell

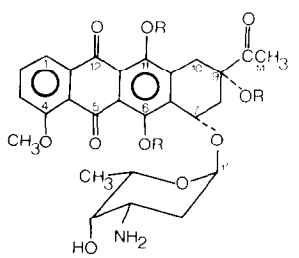

Fig. 1. Structure of daunorubicin (R = H) and derivatives (R = CH_3 or H, as indicated in the text).

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