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# Inhibition of rat brain glutamate decarboxylase activity by salicylate *in vitro*

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Salicylate inhibited rat brain glutamate decarboxylase activity *in vitro*. The mechanism of the inhibition appeared to involve irreversible combination of the drug with the enzyme protein. Salicylate also decreased the incorporation of radioactivity from L-glutamic acid-$^{14}$C(U) into $\gamma$-aminobutyrate in preparations of rat brain, an effect consistent with an inhibition of glutamate decarboxylase activity by the drug.

SALICYLATE inhibits *Escherichia coli* glutamate decarboxylase *in vitro* (Gould, Huggins & Smith, 1963). In the present work the effects of salicylate on glutamate decarboxylase activity and the transfer of radio carbon from labelled glutamate to $\gamma$-aminobutyrate in rat brain preparations have been investigated.

## Experimental

### RAT BRAIN PREPARATION

Male Wistar rats, 200 to 300 g, maintained on M.R.C. cube diet no. 41, were killed by cervical fracture. The brain was removed, placed in the appropriate ice-cold buffer and weighed on a torsion balance. Two types of homogenate were prepared. The first, used for the determination of glutamate decarboxylase activity, was made in sufficient 0·05 M potassium phosphate buffer, pH 5·9, with a Dounce homogeniser, to give either a 20% w/v or a 40% w/v homogenate. The other, used in the radioactive experiments, was prepared in a similar manner using 0·01M potassium phosphate buffer, pH 7·4 to give a 33% w/v homogenate.

### MEASUREMENT OF GLUTAMATE DECARBOXYLASE ACTIVITY

The method was based on that described by Tashain (1961). Assays were made in a standard Warburg apparatus at 37°. All solutions were prepared in 0·05M potassium phosphate buffer, pH 5·9. Two ml of the 20% w/v brain homogenate were placed in the main compartment of the Warburg flask and 1 ml of 0·3M L-glutamic acid containing 200 $\mu$g of pyridoxal phosphate, and the salicylate, when present, was placed in the side-arm. The manometers were flushed with nitrogen for 6 min, then equilibrated at 37° for a further 6 min. and the reaction started by tipping the glutamate solution from the side-arm. The $CO_2$ evolved was measured at 5 min intervals for 20 min and the values used to calculate the initial rate of the reaction. This was corrected for dissolved $CO_2$ by recalculation of the flask constants by the method described by Umbreit (1957).

Further experiments were made to define the mechanism of the inhibitory action of salicylate. Varying amounts of the 40% w/v brain homogenate were exposed to 150 mM salicylate for 30 min at 37° before

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the reaction was started by adding the glutamate solution to give a final volume of 3 ml.

RADIOACTIVE EXPERIMENTS

Portions (400 $\mu$l) of the 33% w/v brain homogenate were added to a solution containing 5 $\mu$c (0·5 $\mu$mole) of radioactive glutamate and the salicylate, when present, in 100 $\mu$l of 0·01M potassium phosphate buffer, pH 7·4. The L-glutamic acid-$^{14}$C(U) was obtained from the Radiochemical Centre, Amersham, Bucks. The mixtures were incubated at 37° with shaking and 100 $\mu$l samples removed at intervals of 5, 15, 30 and 60 min. After the addition of 400 $\mu$l of boiling ethanol, the samples were centrifuged and the radioactive substances present in the supernatants separated by two dimensional paper chromatography, visualised by radioautography and the $^{14}$C measured by the techniques described by Smith & Moses (1960).

## Results

The results in Table 1 show that salicylate inhibits glutamate decarboxylase activity in the rat brain preparation and that the degree of inhibition increased with the salicylate concentration.

TABLE 1. EFFECT OF SALICYLATE ON RAT BRAIN GLUTAMATE DECARBOXYLASE ACTIVITY

| Final concentration of salicylate (mM) | Inhibition (%) |
|---|---|
| 15 | 16 |
| 25 | 31 |
| 40 | 50 |
| 50 | 59 |
| 75 | 87 |
| 100 | 92 |
| 150 | 99 |

Each value represents the mean of six determinations. In the control experiments 26·0 $\mu$l of $CO_2$ were evolved during 20 min.

Table 2 shows the effect of exposing the rat brain preparation of glutamate decarboxylase to 150 mM salicylate before the reaction was started by the addition of the substrate. If the inhibitory effect of salicylate was reversible, then the observed degree of inhibition should have been

TABLE 2. EFFECT OF PRE-INCUBATING ENZYME PREPARATION WITH 150mM SALICYLATE

| Final salicylate conc. in reaction mixture (mM) | Control rate ($\mu$l $CO_2$/20 min) | Theoretical inhibition (%) | | Observed inhibition (%) |
|---|---|---|---|---|
| | | Reversible | Irreversible | |
| 25 | 12·8 | 31 | 99 | 99 |
| 50 | 26·0 | 59 | 99 | 99 |
| 100 | 50·3 | 92 | 99 | 99 |

determined by the final salicylate concentration in the reaction mixture. However, it was found that the inhibition persisted at the level induced by the salicylate concentration to which the enzyme preparation had been

exposed before the start of the reaction. The subsequent dilution of the 150 mM salicylate in the final reaction mixture therefore did not affect the degree of inhibition. This result indicated that salicylate causes an irreversible inhibition of the glutamate decarboxylase in the rat brain preparation.

The results in Table 3 show that the rat brain preparation incorporated radiocarbon from the labelled glutamate into γ-aminobutyrate and into glutamine showing that glutamate decarboxylase and glutamine synthetase activities were present. Salicylate caused a decreased formation of both labelled intermediates at all the times studied. Radioactivity also occurred

TABLE 3. EFFECTS OF 10mM SALICYLATE ON THE DISTRIBUTION OF RADIOACTIVITY FROM L-GLUTAMIC ACID-$^{14}$C(U) INTO THE SOLUBLE METABOLIC INTERMEDIATES OF THE RAT BRAIN PREPARATION

Results given as counts per min × $10^{-2}$ of $^{14}$C.

| Soluble intermediate | 5 min | | 15 min | | 30 min | | 60 min | |
|---|---|---|---|---|---|---|---|---|
| | Control | Salicylate | Control | Salicylate | Control | Salicylate | Control | Salicylate |
| γ-Aminobutyrate | 47·2 | 31·1 | 139·6 | 64·9 | 217·4 | 110·2 | 203·5 | 104·3 |
| Glutamine .. | 1·5 | 0·6 | 3·1 | 0 | 4·6 | 2·8 | 2·8 | 0·6 |
| α-Oxoglutarate .. | 55·0 | 69·9 | 52·6 | 38·1 | 18·7 | 33·2 | 15·6 | 22·5 |
| Tricarboxylic cycle acids (succinate; fumarate: malate: citrate) .. | 12·3 | 14·2 | 13·9 | 27·9 | 19·1 | 19·4 | 31·7 | 26·1 |
| Aspartate .. | 12·3 | 9·8 | 17·3 | 28·3 | 36·2 | 43·6 | 40·2 | 60·6 |

in α-oxoglutarate, various acids of the tricarboxylic acid cycle and in aspartate, showing that glutamate carbon had been transferred via α-oxoglutarate to the Krebs cycle. The aspartate was presumably formed by transamination of oxaloacetate. The salicylate did not cause either marked or consistent effects on the distribution of the isotope in these fractions except that the formation of labelled aspartate was increased at the later time intervals.

## Discussion

Salicylate inhibits glutamate decarboxylase activity *in vitro* in the rat brain preparation used. The results of the radioactive experiments confirm this because the transfer of radioactivity from labelled glutamate to γ-aminobutyrate is markedly decreased in the presence of the drug. The radioactive work also provides evidence that salicylate interferes with the conversion of glutamate to glutamine, and Messer (1958) has shown that salicylate inhibits glutamine synthetase activity in mammalian brain.

The mechanism of the inhibitory action of salicylate on the rat brain glutamate decarboxylase is irreversible and is the same as that reported for the *E. coli* enzyme (Gould, Huggins & Smith, 1963). A possible mechanism is that salicylate combines with free amino-groups in the enzyme protein because it has been shown that the drug combines with ε-amino-groups in bovine albumin (Davison & Smith, 1961).

The finding that salicylate inhibits rat brain glutamate decarboxylase activity *in vitro* by an irreversible mechanism may have important implications *in vivo*. The drug interferes with other enzymes, but the inhibitions

are reversible. Thus many dehydrogenases are inhibited by salicylate but the mechanism involves competition with the pyridine nucleotide coenzymes (Hines & Smith, 1964) and may be reversed by the further addition of coenzyme even after the inhibition has become established (Smith, Bryant & Hines, 1964). Salicylate inhibits aminotransferase enzymes *in vitro* by competing with the amino- and oxo-acid substrates (Gould, 1964) and theoretically an *in vivo* inhibitory action of any particular salicylate concentration on these enzymes will disappear if the substrate concentrations increase. In addition, the effects of salicylate on these reversibly inhibited enzymes should progressively decrease as salicylate is removed from an *in vivo* system. This consideration does not apply to glutamate decarboxylase. Once the inhibitory effect of salicylate on the decarboxylase has become established it will not be altered by the subsequent removal of the drug, and presumably will persist until the enzyme protein is renewed. Thus it may cause a prolonged toxic effect in a patient poisoned with salicylate even when the drug has been removed from the body by renal excretion or haemodialysis.

If salicylate inhibits glutamate decarboxylase activity in the human brain, an expected result would be a decreased conversion of glutamate to $\gamma$-aminobutyrate. Altered ratios of the two amino-acids may occur in various regions of the central nervous system. There is experimental evidence (Way & Sutherland, 1963) that glutamate is a neuronal excitant whereas $\gamma$-aminobutyrate has an opposite action. A high glutamate: $\gamma$-aminobutyrate ratio resulting from an inhibition of glutamate decarboxylase activity, could cause a stimulation of the central nervous system and Roberts, Rothstein & Baxter (1958) have reported that a decrease in the brain glutamate decarboxylase activity produced convulsions in mice. Convulsions occur frequently in salicylate poisoning in man (Gross & Greenberg, 1948) and they may be mediated via an inhibitory action of the drug on glutamate decarboxylase in the brain.

## References

Davison, C. & Smith, P. K. (1961). *J. Pharmacol.*, **133**, 161–170.
Gould, B. J. (1964). Ph.D. Thesis, University of London.
Gould, B. J., Huggins, A. K. & Smith, M. J. H. (1963). *Biochem. J.*, **88**, 346–349.
Gross, M. & Greenberg, L. A. (1948). *The Salicylates*, p. 167. New Haven: Hill House Press.
Hines, W. J. W. & Smith, M. J. H. (1964). *Nature, Lond.*, **201**, 192.
Messer, M. (1958). *Aust. J. exp. Biol. med. Sci.*, **36**, 65–76.
Roberts, E., Rothstein, M. & Baxter, C. F. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 796–802.
Smith, M. J. H. & Moses, V. (1960). *Biochem. J.*, **76**, 579–585.
Smith, M. J. H., Bryant, C. & Hines, W. J. W. (1964). *Nature, Lond.*, **202**, 96–97.
Tashain, R. E. (1961). *Metabolism*, **10**, 393–402.
Umbreit, W. W. (1957). In *Manometric Techniques*, Editors: Umbreit, W. W., Burris, R. H. & Stauffer, J. P., p. 30. Minneapolis: Burgess Publishing Co.
Way, E. L. & Sutherland, V. C. (1963). *Anesthesiology*, **24**, 543–562.