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The mechanism of the inhibitory action of salicylate on glutamyl-transfer ribonucleic acid synthetase *in vitro*

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Salicylate inhibits glutamyl-transfer ribonucleic acid synthetase by simple competitive inhibition with respect to glutamate and mixed competitive-non-competitive inhibitions with ATP and tRNA. It is suggested that competition between salicylate and amino-acid explains the variable inhibition of the synthetases by the drugs *in vitro*. The action of salicylate is consistent with a mechanism for glutamyl-tRNA formation involving a concerted interaction of all three substrates. The drug may selectively interfere with protein synthesis *in vivo*.

Salicylate has been shown to inhibit the synthesis of aminoacyl-tRNAs (Burleigh & Smith, 1971). The most sensitive enzyme is glutamyl-tRNA synthetase. The present paper describes kinetic experiments designed to elucidate the mechanism of inhibition by using fixed concentrations of two of the substrates and varying the third in the presence of different concentrations of salicylate. Changes in the Mg^{2+}: ATP ratio in the reaction mixtures (Novelli, 1967) and the possible binding of salicylate to tRNA were also investigated; Burleigh & Smith (1970) gave a preliminary account.

MATERIALS AND METHODS

Materials

L-[$U^{14}C$]Glutamic acid (260 mCi/mmol) was obtained from the Radiochemical Centre, Amersham; ATP (sodium salt), L-glutamic acid, tRNA from Bakers yeast (Type III) and from calf liver (Type IV) and ribonuclease-A from bovine pancreas (salt-free) from the Sigma Chemical Company; PPO and POPOP from Packard Instrument Co. Inc; Sephadex G-25 from Pharmacia; Visking dialysis tubing (8/32 inch inflated diameter) from Scientific Instruments Centre, London.

Methods

The preparation of a 105 000 *g* supernatant and its passage through Sephadex G-25, the extraction of aminoacyl-tRNA, the measurement of radioactivity and the estimation of tRNA were according to Burleigh & Smith (1971).

Binding of salicylate to tRNA

This was studied by an equilibrium dialysis method (McArthur & Smith, 1969) using either 0·1M phosphate buffer (pH 7·4) or 0·1M tris-HCl (pH 7·4 at 20°) as dialysing medium. The Visking tubing was soaked in two changes of distilled water for 20 min before use. The tRNA solution (1 ml containing 500 μg of calf liver tRNA in buffer) was placed inside each dialysis sac at the beginning of the experiment and dialysed against 3 ml of buffer, containing initial salicylate concentrations ranging from 0–10 mM, for 24 h at 20° with shaking on a Luckman Rotary Shaker at 100

cycles/min. At the end of the experiment the unbound salicylate was measured in the fluids outside the dialysis sacs with an Aminco-Bowman spectrophotofluorometer at an excitation wavelength of 294 nm and a detection wavelength of 413 nm.

RESULTS

Kinetic experiments

In each experiment the activity of glutamyl-tRNA synthetase in the 105 000 *g* supernatant was estimated by measuring the incorporation of radioactivity from labelled glutamate into aminoacyl-tRNA at 2·0, 3·0 or 3·5 min at 37°. The reaction ceased to be linear after 4 min. The initial rates (v) were calculated from the slopes of the activity/time curves. Separate experiments were made with the three substrates; glutamate, ATP and tRNA, in which the concentrations of two of these were fixed and the third was varied in the presence of different concentrations of salicylate. Primary plots (1/v against 1/S) were constructed (Lineweaver & Burk, 1934) and the slopes from the primary plots were plotted against salicylate concentration.

Fig. 1 shows the results of varying the concentration of glutumate and salicylate in the presence of constant concentrations of ATP and tRNA. Figs 2 and 3 give the results of similar experiments in which the concentrations of salicylate and either ATP or tRNA were varied and those of glutamate and either tRNA or ATP were fixed.

The results in Fig. 1 show that the primary plots intersect on the 1/v axis and that the secondary plot of the slopes against salicylate concentration is linear. They are consistent with a mechanism of inhibition involving simple competition between

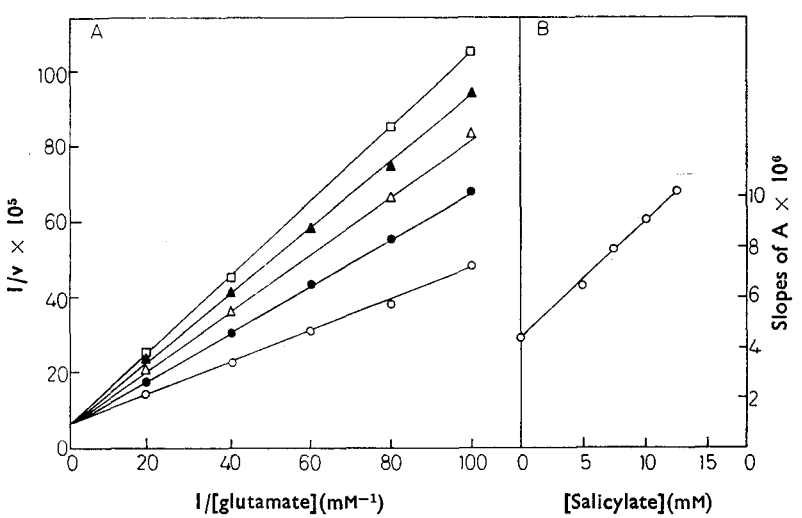

FIG. 1. Glutamyl-tRNA synthetase, kinetics with constant concentrations of ATP and tRNA. Each reaction mixture contained, in a total volume of 1·0 ml, 100 μmol tris-HCl (pH 7·2 at 37°); 10 μmol $MgCl_2$; 2 μmol ATP; 500 μg yeast tRNA; 0·4 ml 105 000 *g* supernatant (7·5 mg protein); sufficient of a mixture of KCl and potassium salicylate to yield final salicylate concentrations ranging from 0–12·5 mM and a constant K^+ concentration of 15 mM; and mixtures of L-[^{14}C]-glutamate and L-glutamate to give total glutamate concentrations ranging from 0·01 to 0·05 mM and specific activities from 200 to 20 mCi/mmol. The reaction was started by the addition of the supernatant and duplicate tubes were incubated at 37° for, 2, 3 or 3·5 min. Zero time counts have been subtracted from the experimental values which were calculated as counts/min mg^{-1} RNA isolated corrected to a specific activity of radioactive glutamate of 100 mCi/mmol. A. ○—○ Control; ●—● 5·0 mM salicylate; △—△ 7·5 mM salicylate; ▲—▲ 10·0 mM salicylate; □—□ 12·5 mM salicylate. B. Secondary plot of slopes of A.

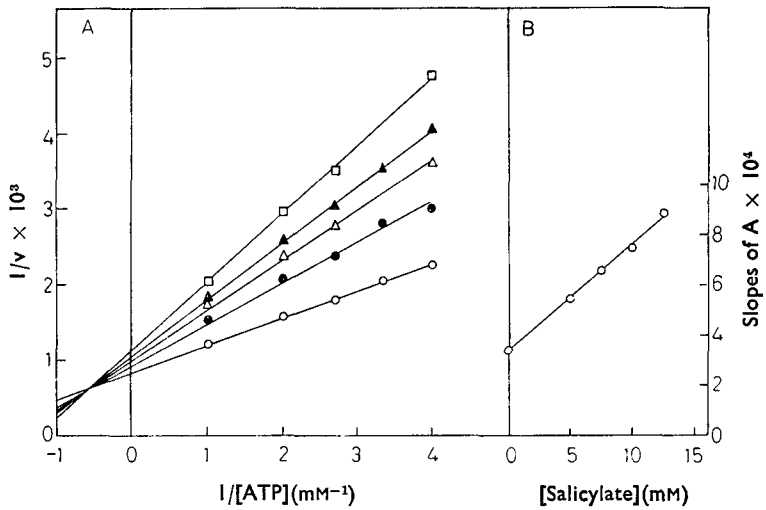

FIG. 2. Glutamyl-tRNA synthetase, kinetics with constant concentrations of glutamate and tRNA. Experimental conditions similar to Fig. 1 except that the concentrations of ATP in the reaction mixtures ranged from 0·25–1·00 mM and the L-[^{14}C]glutamate plus L-glutamate concentration was 0·04 mM (specific activity 38·5 mCi/mmol). A. ○—○ Control; ●—● 5·0 mM salicylate; △—△ 7·5 mM salicylate; ▲—▲ 10·0 mM salicylate; □—□ 12·5 mM salicylate. B. Secondary plot of slopes of A.

glutamate and salicylate. In contrast the primary plots in Figs 2 and 3 intersect above the 1/S axis and to the left of the 1/v axis indicating mixed competitive-non-competitive mechanisms of inhibition between salicylate and either ATP or tRNA. All the secondary plots were linear and the values for K_I calculated from the intercepts on the salicylate concentration axis were 8·7, 8·3 and 8·6 respectively.

The effect of varying the Mg^{2+}: ATP ratio

The curves obtained either in the absence or the presence of 10 mM salicylate were similar in shape and position showing that the drug does not inhibit the synthetase activity by interacting with magnesium ions. It also follows that the differential inhibition of aminoacyl-tRNA synthetases by salicylate is not due to an interaction between Mg^{2+} and the drug although the enzymes have different Mg^{2+}:ATP optima.

Binding of salicylate to tRNA

One possible explanation of the inhibitory effect of salicylate on the synthetase activity is that the drug binds to the tRNA. The equilibrium dialysis experiments revealed that no such binding was observed in a tris-HCl medium and a similar result was obtained with the phosphate medium.

DISCUSSION

The results show that salicylate inhibits the activity of glutamyl-tRNA synthetase by a simple competitive mechanism involving glutamate and mixed competitive-non-competitive mechanisms with ATP and tRNA. The drug did not act either by altering the effective concentration of Mg^{2+} or by binding to the tRNA.

There are several implications of the results. One concerns the mechanism for the biosynthesis of aminoacyl-tRNA. This has been suggested to occur in two stages, activation of the amino-acids (Hoagland, 1955) and their transfer to the tRNA (Hoagland, Zamecnik & Stephenson, 1957). Equations 1 and 2 show the inter-

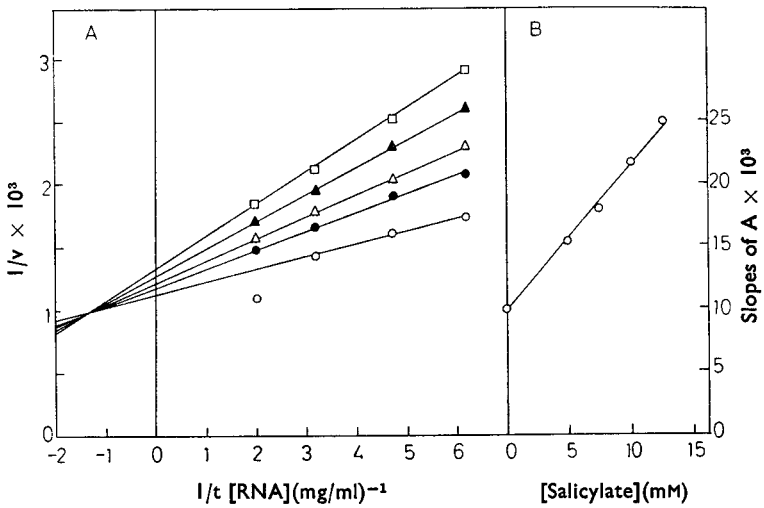

FIG. 3. Glutamyl-tRNA synthetase, kinetics with constant concentrations of glutamate and ATP. Experimental conditions similar to Fig. 2 except that the concentration of ATP in each reaction mixture was 2 mM and the yeast tRNA was replaced by sufficient calf liver tRNA to give final tRNA concentrations (calf + endogenous) ranging from 162–504 μg/ml. At the end of the incubation sufficient calf liver tRNA was added to give a total of 1 mg per tube. A. ○—○ Control; ●—● 5·0 mM salicylate; △—△ 7·5 mM salicylate; ▲—▲ 10·0 mM salicylate; □—□ 12·5 mM salicylate. B. Secondary plot of slopes of A.

mediates involved and equation 3 represents the over-all reaction. The symbols refer to the following: AA is amino-acid, PP_i is pyrophosphate, E is enzyme, E(AA — AMP) is enzyme-bound aminoacyl-adenylate and AA-tRNA is aminoacyl-transfer ribonucleic acid.

$$AA + ATP + E \rightleftharpoons E(AA - AMP) + PP_i \quad .. \quad .. \quad (1)$$

$$E(AA - AMP) + tRNA \rightleftharpoons AA - tRNA + AMP + E \quad .. \quad (2)$$

$$AA + ATP + tRNA \rightleftharpoons AA - tRNA + AMP + PP_i \quad .. \quad (3)$$

The main evidence in favour of a two-stage mechanism is the isolation of radioactive enzyme-bound aminoacyl-adenylates from reaction mixtures containing ATP, substrate amounts of purified enzymes from either bacteria or mammalian tissues and either [^{14}C]isoleucine, [^{14}C]tryptophan or [^{14}C]threonine (Wong & Moldave, 1960; Norris & Berg, 1964; Allende, Allende & others, 1964). The results of experiments using carboxy-[^{18}O]tryptophan (Hoagland, Zamecnik & others, 1957) are consistent with the two-stage mechanism but do not exclude a single concerted reaction as in equation 3 (Boyer, 1960). The ATP-pyrophosphate exchange reaction has been considered to reflect equation 1 but it has been observed that in mammalian systems glutamate does not promote this exchange in the absence of tRNA (Deutscher, 1967) and a similar result occurs with several amino-acids and bacterial aminoacyl-tRNA synthetases (Mitra & Mehler, 1967). The participation of E(AA — AMP) as an obligatory intermediate was questioned by Loftfield & Eigner (1969) on the basis of their data on the formation of aminoacyl hydroxamates. These workers (Loftfield & Eigner, 1968) also showed that ammonia and imidazole inhibit the ATP-pyrophosphate exchange but stimulate the esterification of tRNA. Salicylate inhibits the exchange reaction promoted by leucine and phenylalanine but does not affect the incorporation of these amino-acids into aminoacyl-tRNA (Burleigh & Smith, 1971).

The results of the present work (Figs 1–3) are consistent only with a mechanism for the action of glutamyl-tRNA synthetase involving a concerted interaction of the three substrates leading to the formation of a quaternary complex. A two-stage reaction mechanism would necessitate the inhibition by salicylate being uncompetitive with respect to tRNA (Fromm, 1967). Although our work has been restricted to the glutamate enzyme similar considerations could apply to the other aminoacyl-tRNA synthetases. The formation of enzyme-bound aminoacyl-adenylates from certain amino-acids, reflected by the ATP-pyrophosphate exchange, may occur as a side reaction.

The proposed mechanism of inhibition of the aminoacyl-tRNA synthetases by salicylate, involving simple competition with the amino-acid substrates, suggest that the drug may selectively interfere with protein biosynthesis *in vivo*. Whether or not the incorporation of a particular amino-acid into tissue proteins were inhibited would depend on the endogenous concentration of the amino-acid, the K_m of the corresponding synthetase and the K_I of the enzyme for salicylate.

Some proteins are relatively rich in certain amino-acids. An example is the immunoglobulins which contain more tryptophan than other proteins (see Press & Piggot, 1967). If the incorporation of tryptophan plays a part in regulating the biosynthesis of immunoglobulins *in vivo* then salicylate may act as a selective inhibitor by affecting tissues, such as lymphocytes, which are principally concerned with the manufacture of antibodies. A further possibility in the same category is pyrrolidone-γ-carboxylate, which is a common *N*-terminal group in antibodies and may also act as an initiator of immunoglobulin biosynthesis (Wikler, Titani & others, 1967).

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