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The mechanism of inhibition by salicylate of the pentose phosphate pathway in the human red cell

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Salicylate inhibits the pentose phosphate pathway of glucose metabolism in human red cell haemolysates. The site of inhibition is concerned with glucose 6-phosphate and 6-phosphogluconate dehydrogenases and the mechanism of inhibition involves competition with NADP.

SALICYLATE exerts multiple effects on glucose metabolism in the human red cell. The drug interferes with glycolytic reactions and with the pentose phosphate pathway, the latter route of glucose catabolism being the more sensitive (Sturman & Smith, 1966). One possible explanation of these findings is that salicylate inhibits the activities of glucose 6-phosphate and 6-phosphogluconate dehydrogenases by a mechanism involving competition with the pyridine nucleotide coenzyme, NADP. It has been shown that salicylate inhibits the activities of several other dehydrogenases, including malate, lactate and isocitrate, by competing with either NAD or NADP (Hines & Smith, 1964) and that the overall activity of the pentose phosphate pathway is largely governed by the availability of NADP (Couri & Racker, 1959).

The present work is concerned with studies of the effects of salicylate on a haemolysate of human red cells, incubated under conditions in which the supply of NADP was not a limiting factor in determining the proportion of glucose metabolized by the pentose phosphate pathway. In addition the effects of salicylate on glucose 6-phosphate and 6-phosphogluconate dehydrogenase preparations from human red cells were investigated with special reference to the mechanism of inhibition.

Experimental

RED CELL HAEMOLYSATES

Preparation. Venous blood was collected from healthy adults and heparin used as anticoagulant. The white cells were removed by filtering through cotton wool and washing three times with 0·15M sodium chloride (Buchanan, 1960). The red cells were haemolysed by adding an equal volume of water and then rapidly freezing and thawing the mixture.

OPTIMUM CONCENTRATION OF NADP

Incubations were carried out at 37° in stoppered Warburg flasks, the side arms of which contained 0·5 ml of 50% (w/v) trichloroacetic acid. The centre well contained 0·2 ml of 20% (w/v) potassium hydroxide and the main compartment 2 ml of 0·1M glycylglycine buffer, pH 7·4, containing [$^{14}C_1$] glucose (specific activity 1·39 μc/mg), ATP, NAD, Mg^{2+} and inorganic phosphate ions to give final concentrations of 1 mM, 1 mM, 0·3 mM, 1·5 mM and 2 mM respectively (Chapman, Hennessey, Walters-dorph & others, 1962) and NADP in final concentrations ranging from 0·01

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to 5 mM. The reaction was started by the addition of 2 ml of haemolysate. After an incubation period of 1 hr, the trichloroacetic acid was added from the side arm and the incubation continued for a further hour to ensure that all $^{14}CO_2$ was absorbed in the centre well. The suspension in the main compartment was removed, centrifuged at 2000 *g* for 30 min to separate the cell debris, and the supernatant used for the determinations. Glucose was separated from lactate and phosphate compounds by chromatography on Whatman No. 4 paper using butanol–propionic acid–water (3:2:2), and located by a Nuclear-Chicago Actigraph scanner. The amounts of glucose initially present were measured in corresponding mixtures to which the trichloroacetic acid was added at zero time. Portions of the solution in the centre well were dried on Whatman No. 4 paper. All radioactive counting was performed directly on paper with a Packard Tri-Carb Liquid Scintillation Spectrometer, using as phosphor 15 ml of 0·4% 2,5-diphenyloxazole and 0·01% 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene in toluene.

EFFECTS OF SALICYLATE ON [$^{14}C_1$] GLUCOSE METABOLISM IN HAEMOLYSATES

Similar experiments were made in the presence of salicylate, added to the reaction mixtures to give final concentrations ranging from 5 to 500 mM except that the final concentration of NADP present was left constant at 1 mM.

DEHYDROGENASE EXPERIMENTS

Preparation. To 20 ml of red cell haemolysate was added 20 ml of 8% w/v DEAE cellulose (1 m-equiv./g) suspension and the mixture stirred for 20 min at 4°. The supernatant, principally haemoglobin, was removed by centrifuging for 15 min at 1900 *g* and any residual haemoglobin was removed by washing the adsorbent 5 times with a total of 1 litre of 0·003 M potassium phosphate buffer, pH 7·0. The enzyme proteins were desorbed from the cellulose with 10 ml of 0·5M potassium chloride and stirred for 1 hr in an ice bath. The supernatant was removed by centrifuging for 15 min at 1900 *g*. Serum albumin in a final concentration of 10 mg/ml was added immediately to this supernatant in order to stabilize the dilute enzymes. This procedure was repeated once more with the adsorbent and the supernatants combined. The resulting solution was used as the enzyme preparation in all subsequent work.

Effects of salicylate on dehydrogenase activities. Glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities were determined by the techniques described by Kornberg & Horecker (1955) and Horecker & Smyrniotis (1955). The assays were carried out in 0·05M glycylglycine buffer, pH 7·4, at 25° and followed by measuring the increase in optical density at 365 mμ (reduction of NADP to $NADPH_2$), in a Unicam SP800 recording spectrophotometer. Measurements of extinction were made at 365 mμ, and not at 340 mμ, to avoid interference caused by the absorption of the salicylate at the lower wavelength. The initial rates, determined from the tracings obtained with an external recorder, were obtained

in either the absence or the presence of 20 or 50 mM salicylate and with NADP concentrations ranging from 0·01 to 1 mM.

Results

The results, given in Fig. 1, clearly show that maximum activity of the pentose phosphate pathway, assessed by the amount of $^{14}CO_2$ produced and by the proportion of the [$^{14}C_1$] glucose converted to $^{14}CO_2$, occurred

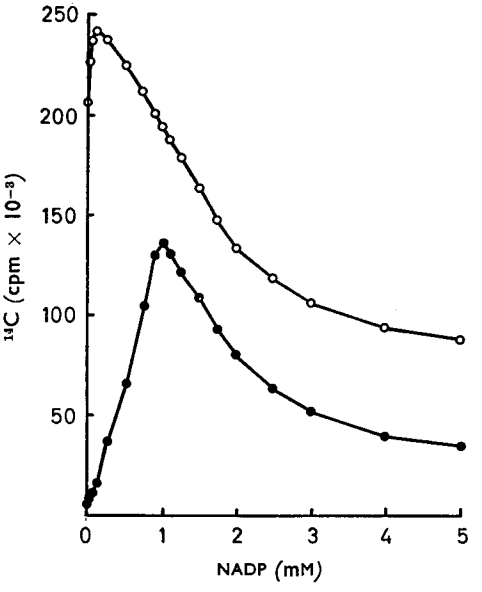

FIG. 1. Effects of NADP concentration on [$^{14}C_1$] glucose utilization and $^{14}CO_2$ production by human red cell haemolysates. ○, [$^{14}C_1$] glucose utilized; ●, $^{14}CO_2$ produced.

after the addition of 1 mM NADP. This concentration of NADP was therefore added to all the subsequent reaction mixtures used to study the effects of salicylate on glucose metabolism in the red cell haemolysates.

Table 1 shows that the addition of increasing concentrations of salicylate reduce both the utilization of the [$^{14}C_1$] glucose and the production of

TABLE 1. EFFECTS OF SALICYLATE ON THE UTILISATION OF [$^{14}C_1$] GLUCOSE AND PRODUCTION OF $^{14}CO_2$ BY HUMAN RED CELL HAEMOLYSATES. The results represent the means of duplicate experiments. Radioactivity is expressed as disintegrations per min (dpm) × 10^{-3}

Salicylate concentration (mM)	[$^{14}C_1$] Glucose utilized	$^{14}CO_2$ produced	% [$^{14}C_1$] Glucose converted to $^{14}CO_2$
0	421	281	66·8
5	386	272	70·5
10	368	267	72·5
20	325	252	77·4
50	191	158	82·8
100	115	103	90·0
200	17	16	92·9
500	2	2	98·7

$^{14}CO_2$ by the haemolysates. However, the percentage of [$^{14}C_1$] glucose converted to $^{14}CO_2$ rose as the salicylate concentration was increased. Thus in the presence of the 1 mM NADP, the pentose phosphate pathway is less sensitive to the inhibitory action of salicylate than the glycolytic pathway.

TABLE 2. EFFECTS OF SALICYLATE ON THE GLUCOSE 6-PHOSPHATE DEHYDROGENASE ACTIVITY OF HUMAN RED CELL HAEMOLYSATES. The results represent the means of duplicate experiments and are expressed as percentage inhibitions

	Salicylate	
NADP concentration (mM)	20 mM	50 mM
0·01	41·2	65·2
0·025	25·7	48·5
0·05	18·8	31·3
0·075	14·2	30·8
0·10	13·1	28·2
0·25	9·0	18·7
0·50	5·5	12·4
0·75	4·0	9·4
1·00	0·0	7·2

TABLE 3. EFFECTS OF SALICYLATE ON THE 6-PHOSPHOGLUCONATE DEHYDROGENASE ACTIVITY OF HUMAN RED CELL HAEMOLYSATES. The results represent the means of duplicate experiments and are expressed as percentage inhibitions.

NADP concentration (mM)	Salicylate (50 mM)
0·01	20·8
0·025	14·6
0·05	9·3
0·075	7·1
0·10	5·5

The results in Tables 2 and 3 show that salicylate inhibits the activities of both glucose 6-phosphate and of 6-phosphogluconate dehydrogenases in the red cell extract and that the degree of inhibition is reduced by the presence of increasing concentrations of NADP. These data suggested that the mechanism of inhibition of the dehydrogenases by salicylate involved a reversible competition with the coenzyme. More acceptable proof that this is the correct mechanism was obtained by graphically representing the data by the method of Hunter & Downs (1945). The resulting plots (Figs 2 and 3) revealed a straight line relationship dependent on NADP concentration. This is the accepted criterion for a reversible competitive inhibition (Dixon & Webb, 1964).

Discussion

The present work shows that the supply of NADP influences the pattern inhibition of glucose metabolism by salicylate. When the coenzyme is present in limited amounts then salicylate inhibits the pentose phosphate pathway to a greater extent than the glycolytic pathway (Sturman & Smith, 1966) whereas the reverse occurs when the supply of NADP is no longer limiting (Table 1). These observations suggested that salicylate interfered

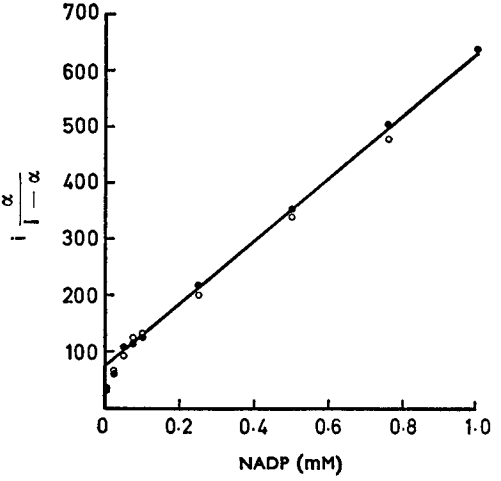

FIG. 2. Inhibition of glucose 6-phosphate dehydrogenase of human red cell haemolysates by salicylate plotted by the method of Hunter & Downs (1945). ○, data obtained in presence of 20 mM salicylate; ●, data obtained in presence of 50 mM salicylate. Values of kinetic constants from plot: K_I = 70 mM, K_m = 0·12 mM.

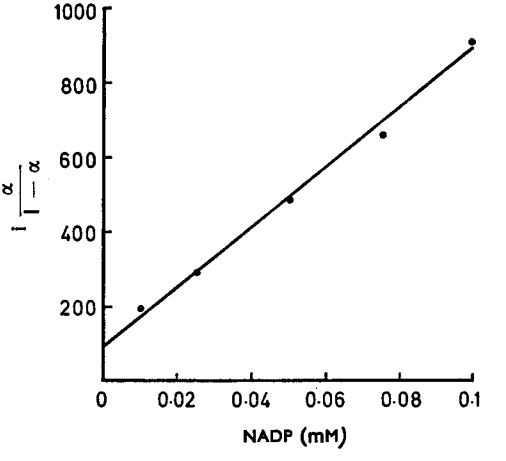

FIG. 3. Inhibition of 6-phosphogluconate dehydrogenase of human red cell haemolysates by salicylate plotted by the method of Hunter & Downs (1945). Data obtained in presence of 50 mM salicylate. Values of kinetic constants from plot: K_I = 100 mM, K_m = 0·011 mM.

with the NADP-dependent dehydrogenases concerned with the production of $^{14}CO_2$ from [$^{14}C_1$] glucose. The results in Tables 2 and 3 show that salicylate inhibits the activities of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in extracts prepared from the red cell haemolysates. In addition the degree of inhibition produced by a given salicylate concentration varied with the NADP concentration. From the experimental data were prepared graphical plots (Figs 2 and 3) which showed

that the mechanism of inhibition of the two dehydrogenases by salicylate involved a reversible competition with NADP.

The inhibitor constants (K_I) for both enzymes were also determined from the plots. They indicate that salicylate is a stronger inhibitor (K_I = 70 mM) of glucose 6-phosphate dehydrogenase than of 6-phosphogluconate dehydrogenase (K_I = 100 mM). The K_I values show that salicylate is a relatively weak inhibitor of both dehydrogenase activities *in vitro*. This does not necessarily mean that salicylate, in the concentration ranges observed either during therapy (2 to 3 mM) or during acute poisoning (3 to 10 mM) will not affect the dehydrogenase activities, and hence carbohydrate metabolism, in circulating human red cells. There is experimental evidence that 5 mM salicylate preferentially inhibits the pentose phosphate pathway in human erythrocyte suspensions (Sturman & Smith, 1966). It is also possible to calculate, from the data in Figs 2 and 3, that while 2 to 10 mM salicylate would produce little, if any, inhibition of 6-phosphogluconate *in vitro* these salicylate concentrations would inhibit the glucose 6-phosphate dehydrogenase activity between 5 and 20%. As the mechanism of inhibition of the dehydrogenases by salicylate involves a reversible competition with the coenzyme, the degree of inhibition *in vivo* would depend, at least in part, on the intra-erythrocytic concentrations of NADP and these are relatively low, a range of 0·013 to 0·017 mM having been found (Bartlett, 1959).

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