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# EFFECTS OF SALICYLATE AND γ-RESORCYLATE (2:6-DIHYDROXYBENZOATE) ON PATHWAYS OF GLUCOSE METABOLISM IN THE HUMAN RED CELL

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**Abstract**—Salicylate and γ-resorcylate, in concentrations ranging from 2 to 20 mM, exert multiple effects on the metabolism of radioactive glucose by mature human erythrocytes. The drugs interfere with glycolytic reactions, inhibiting hexokinase and lactate dehydrogenase activities, and with the pentose phosphate pathway by inhibiting glucose-6-phosphate dehydrogenase activity. γ-Resorcylate (10 and 20 mM) but not salicylate, inhibits phosphofructokinase activity.

SALICYLATE and γ-resorcylate have been shown to exert multiple actions on intermediary metabolism in animal tissue preparations. These include an interference with oxidative phosphorylation reactions,[1] reversible inhibition of aminotransferase and dehydrogenase enzymes[2, 3] and an irreversible inhibition of glutamate decarboxylase.[4] The interaction of these inhibitions in tissues, such as liver or kidney, leads to complicated effects on the distribution of radioactivity from labelled substrates into soluble metabolic intermediates.[5] Mature red cells differ from other mammalian tissues because they lack biosynthetic mechanisms for protein, lipids and glycogen and are deficient in certain enzymes of the tricarboxylic acid cycle. The only mechanism for carbon dioxide production from glucose is via the pentose phosphate pathway. Erythrocyte preparations therefore appeared to offer a potentially simpler system for locating possible sites of action of salicylate and γ-resorcylate on the pathways of glucose metabolism. The present work is concerned with a study of the effects of the drugs on the distribution of radioactivity in the soluble metabolic intermediates of mature human red cells incubated with differentially labelled glucose and on certain enzyme activities in haemolysates.

## MATERIAL AND METHODS

### *Materials*

[$^{14}$C] Glucose (sp. act. 730 μc/mg) and [1-$^{14}$C] glucose (sp. act. 166 μc/mg) were obtained from the Radiochemical Centre, Amersham, Bucks.; 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) from the Packard Instrument Co., La Grange, Illinois; nicotinamide adenine dinucleotide (NAD), the reduced form ($NADH_2$), nicotinamide adenine dinucleotide phosphate (NADP), adenosine triphosphate (ATP), sodium pyruvate, glucose-6-phosphate, 6-phosphogluconate, glucose-6-phosphate dehydrogenase, aldolase, triose phosphate

isomerase, α-glycerophosphate dehydrogenase, lactate dehydrogenase and peroxidase from C. F. Boehringer und Soehne, GmbH., Mannheim, Germany; fructose-6-phosphate from the Sigma Chemical Co., St. Louis, Mo.; glucose oxidase and γ-resorcylate from Koch-Light Laboratories Ltd., Colnbrook, Bucks. and all other materials from British Drug Houses Ltd., Poole, Dorset. De-ionised water was used throughout.

*Preparation of red cell suspensions*

Venous blood, collected from healthy adults, was defibrinated by inverting with glass beads in a stoppered bottle for 10 min. The white cells were removed by filtration through cotton wool and washing three times with the buffer to be used in the incubation.[6] The remaining red cells were resuspended in an equal volume of buffer and the composition checked by centrifuging a sample for 30 min at 2000 *g* in a haematocrit tube. The suspensions thus prepared contained 48–52 ml cells/100 ml suspension, and less than fifty leucocytes/μl. The chromatographic experiments were carried out with a solution consisting of seven parts of 0·15 M NaCl and three parts of 0·1 M potassium phosphate, pH 7·4; and the manometric experiments with 0·1 M potassium phosphate, pH 7·4.

*Preparation of red cell haemolysates*

Red cells were prepared as for the suspensions, using 0·15 M NaCl as the washing medium, and resuspended in an equal volume of 0·05 M glycylglycine buffer, pH 7·4. Complete haemolysis was attained by rapidly freezing and thawing three times.

*Chromatographic experiments*

50 μl of the red cell suspension were added to an equal volume of buffer containing about 1 μc of [$^{14}$C] glucose and the salicylate and γ-resorcylate when present. The mixtures were incubated at 37° for 30 min with constant shaking and the reaction stopped by the addition of 400 μl of boiling ethanol. The resulting mixtures were centrifuged for 5 min at 1800 *g*, the supernatant decanted and the sediment re-extracted twice with 200 μl of 50% (v/v) aqueous ethanol. The radioactive compounds in the combined supernatant and washings were separated by two-dimensional paper chromatography, using phenol-water (72% w/v) as the first solvent and butanol-propionic acid-water (45·9:23·4:30·6, by volume) as the second, and their positions on the chromatogram located by exposure to Blue Brand X-ray film (Kodak Ltd.). The radioactivity in each spot was counted directly on the chromatogram by the method of Smith and Moses.[7] The radioactive spots on the chromatogram were identified presumptively by their chromatographic positions and confirmed by co-chromatography with authentic materials in several solvent systems. In addition the phosphate esters were hydrolysed with human prostatic acid phosphatase, prepared by centrifuging the cells from semen and using the supernatant as the source of the enzyme, and re-chromatographed to identify the organic components.

*Manometric experiments*

The standard Warburg constant volume respirometer was used, and flasks with two side arms employed. One side arm contained the glucose substrate (0·5 ml of 0·035 M glucose containing about 1 μc [1-$^{14}$C] glucose) and the other 0·5 ml of 50%

(w/v) trichloroacetic acid for inactivating the enzyme systems at the end of the incubation. The centre well contained 0·2 ml of 20% (w/v) potassium hydroxide to absorb the carbon dioxide produced during the incubation. The main compartment contained 2 ml of the red cell suspension and 1 ml of buffer containing the salicylate and γ-resorcylate, when present. The flasks were attached to manometers, equilibrated at 37° for 15 min, and the apparatus set for oxygen uptake. The reaction was started by tipping the glucose solution from the side arm. The flasks were shaken constantly and the oxygen uptake noted after 3 hr. The trichloroacetic acid was then added from the other side arm and served the dual purpose of inactivating the enzyme systems and acidifying the suspension, thus releasing any dissolved carbon dioxide. The flasks were shaken for a further hour to allow any carbon dioxide so liberated to be absorbed in the centre well.

The suspension in the main compartment was removed, centrifuged at 200 *g* for 30 min to separate the cell debris, and the supernatant used for the determinations. Glucose, lactate and phosphate compounds were separated by chromatography on Whatman No. 4 paper using butanol-propionic acid-water (3:2:2, by vol) and the radioactive compounds were 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 counter, using as phosphor 15 ml of 0·4% PPO and 0·01% dimethyl POPOP in toluene. Total glucose and lactate estimations were performed by the glucose oxidase[8] and lactate dehydrogenase[9] methods respectively on solutions obtained by eluting the appropriate areas of the chromatogram with water.

*Enzyme assays*

The assays were carried out in 0·05 M glycylglycine buffer, pH 7·4, at 37° using coupled systems linked to the oxidation and reduction of pyridine nucleotides, and followed by measuring the changes in optical density at 365 mμ in a Unicam SP800 recording spectrophotometer. Measurements of extinction were made at 365 mμ, and not 340 mμ, to avoid interference caused by the absorption of the drugs at the lower wavelength. The initial rates were determined from the tracings obtained with an external recorder.

Hexokinase was assayed by the method of Chapman *et al.*,[10] phosphofructokinase by the method of Ling *et al.*,[11] lactate dehydrogenase by the method of Kornberg,[12] glucose-6-phosphate dehydrogenase by the method of Kornberg and Horecker [13] and 6-phosphogluconate dehydrogenase by the method of Horecker and Smyrniotis.[14]

## RESULTS

*The effects of salicylate and γ-resorcylate on the distribution of $^{14}C$ in the soluble metabolic intermediates of human red cells*

The radioactivity measured was that present in the soluble intermediates of the cells and the medium. The $^{14}C$ evolved as $CO_2$ or incorporated into substances which were insoluble in aqueous ethanol was not estimated. The results in Table 1 show the amount of $^{14}C$ incorporated into the separated soluble intermediates in the presence and in the absence of the drugs.

TABLE 1. EFFECTS OF SALICYLATE AND γ-RESORCYLATE ON THE INCORPORATION OF RADIOCARBON FROM [$^{14}$C] GLUCOSE INTO THE SOLUBLE METABOLIC INTERMEDIATES OF HUMAN RED CELLS

| Radioactive intermediate | Control | Salicylate (mM) | | | | γ-Resorcylate (mM) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 5 | 10 | 20 | 2 | 5 | 10 | 20 |
| Hexose monophosphates | 1·5 | 1·2 | 0·9 | 1·0 | 0·8 | 1·3 | 1·8 | 8·2 | 15·5 |
| Fructose diphosphate | 6·1 | 4·8 | 4·3 | 4·1 | 4·3 | 4·7 | 3·8 | 5·6 | 8·4 |
| Diphosphoglycerate | 22·5 | 18·4 | 15·9 | 13·6 | 11·4 | 18·1 | 16·7 | 10·5 | 4·3 |
| Monophosphoglycerates | 2·8 | 2·6 | 2·0 | 2·1 | 1·7 | 2·8 | 2·1 | 2·2 | 2·4 |
| Pyruvate | 4·4 | 6·6 | 7·9 | 7·9 | 7·0 | 9·1 | 8·0 | 5·5 | 3·3 |
| Lactate | 17·6 | 16·6 | 15·1 | 14·3 | 13·1 | 15·7 | 12·1 | 8·7 | 3·8 |
| 6-Phosphogluconate | 0·4 | 0·4 | 0·4 | 0·3 | 0·3 | 0·5 | 0·6 | 0·3 | 0 |
| Uridine diphosphoglucose | 2·1 | 0·9 | 1·2 | 1·1 | 1·4 | 1·3 | 1·9 | 1·7 | 3·4 |
| α-Glycerophosphate | 1·3 | 1·2 | 1·3 | 1·2 | 1·4 | 1·4 | 1·2 | 1·2 | 0·7 |
| Residual glucose | 4·2 | 7·1 | 10·3 | 16·3 | 18·1 | 7·6 | 13·7 | 18·0 | 29·0 |
| % Inhibition of glucose utilized | — | 4 | 7 | 15 | 18 | 5 | 12 | 18 | 32 |

The results are expressed as counts/min × $10^{-3}$ of $^{14}$C and represent the mean of two experiments. The total radioactivity in the [$^{14}$C] glucose initially present in each experiment was 82·6 counts/min × $10^{-3}$.

In the control experiments, radioactivity from the labelled glucose was found in intermediates known to be involved in established metabolic sequences in the human red cell. The presence of labelled hexose monophosphates, fructose diphosphate, monophosphoglycerates, phosphoenolpyruvate, pyruvate and lactate is evidence of the glycolytic pathway. The occurrence of $^{14}C$ in phosphogluconate shows that the pentose phosphate oxidative pathway is present and the incorporation of radiocarbon into uridine diphosphoglucose is evidence for the existence of the uridyl transferase system. The formation of relatively large amounts of labelled 2,3-diphosphoglycerate is characteristic of mammalian red cell metabolism since mature erythrocytes contain large pools of this substance which is actively involved in glucose metabolism.

All concentrations of salicylate and γ-resorcylate caused a decreased utilization of labelled glucose on the chromatogram showing that the utilization of the labelled substrate was impaired. This result suggested that both drugs may have interfered with hexokinase activity, the initial step in glucose utilization. The inhibitory effects of salicylate and γ-resorcylate on hexokinase activity in red cell haemolysates are shown in Table 2 where it is seen that the degree of inhibition observed *in vitro* roughly parallels the decrease in the utilization of the labelled glucose found in the experiments recorded in Table 1.

TABLE 2. EFFECTS OF SALICYLATE AND γ-RESORCYLATE ON ENZYME ACTIVITIES IN RED CELL HAEMOLYSATES

| Enzyme | Salicylate (mM) | | | | γ-Resorcylate (mM) | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 | 5 | 10 | 20 | 2 | 5 | 10 | 20 |
| Hexokinase | — | 3 | 8 | 28 | 5 | 12 | 20 | 52 |
| Lactate dehydrogenase | 10 | 12 | 14 | 17 | 7 | 14 | 21 | 36 |
| Phosphofructokinase | — | — | — | — | 43 | 75 | 90 | 100 |

Results are expressed as percentage inhibitions of enzyme activity and represent the mean of four estimations.

A second effect on the radioactive patterns common to salicylate and resorcylate, except for 20 mM γ-resorcylate, was the accumulation of radioactive pyruvate. This result suggested that the drugs interfered with lactate dehydrogenase activity in the red cell. Table 2 shows that an inhibitory action of salicylate and γ-resorcylate could be demonstrated on the enzyme activity in red cell haemolysates.

The presence of γ-resorcylate, (10 and 20 mM) but not of salicylate, caused marked accumulations of radiocarbon in the hexose monophosphate fraction (Table 1). A possible explanation of this result was that γ-resorcylate inhibited phosphofructokinase activity in the red cell and this was confirmed by the *in vitro* results with the enzyme activity in the haemolysed preparation (Table 2).

*The effects of salicylate and γ-resorcylate on the respiration of human red cells*

The results in Table 3 show the effects of the drugs on a number of parameters measured in human red cell suspensions metabolising glucose containing a trace amount of [1-$^{14}C$] glucose. It has been shown that mature human erythrocytes lack a complete tricarboxylic acid cycle.[15] The oxygen consumption, and more specifically, the production of $^{14}CO_2$ from [1-$^{14}C$] glucose, therefore reflect the amounts of glucose metabolised by the pentose phosphate pathway. The contribution of this pathway to

TABLE 3. EFFECTS OF SALICYLATE AND γ-RESORCYLATE ON THE RESPIRATION OF HUMAN RED CELLS

| Drug conc. (mM) | | Total glucose utilized (μmole) | Total lactate produced (μmole) | Radioactive lactate | $O_2$ uptake (μl) | $^{14}CO_2$ | [1-$^{14}$C] glucose utilized | %[1-$^{14}$C] glucose converted to $^{14}CO_2$ | Sp. act. of lactate (dis/min × $10^{-3}$/mg) |
|---|---|---|---|---|---|---|---|---|---|
| Control | 0 | 7·3 ± 0·2 | 16·5 ± 0·7 | 265 ± 6 | 12·6 ± 0·2 | 22·5 ± 0·3 | 434 ± 6 | 5·2 ± 0·2 | 181 ± 3 |
| Salicylate | 5 | 7·3 ± 0·2 | 15·8 ± 0·6 | 260 ± 8 | 12·4 ± 0·3 | 21·3 ± 0·5 | 432 ± 3 | 4·9 ± 0·1 | 183 ± 4 |
| | 10 | 7·1 ± 0·2 | 14·8 ± 0·6 | 246 ± 4 | 10·7 ± 0·5 | 17·4 ± 0·2 | 404 ± 4 | 4·3 ± 0·1 | 184 ± 6 |
| | 15 | 6·9 ± 0·2 | 13·5 ± 0·2 | 227 ± 5 | 9·0 ± 0·2 | 14·4 ± 0·3 | 394 ± 7 | 3·7 ± 0·1 | 187 ± 6 |
| | 20 | 6·4 ± 0·2 | 11·9 ± 0·1 | 201 ± 5 | 7·9 ± 0·2 | 12·4 ± 0·3 | 373 ± 2 | 3·3 ± 0·1 | 187 ± 3 |
| | 25 | 6·2 ± 0·2 | 10·4 ± 0·4 | 182 ± 6 | 5·9 ± 0·1 | 9·0 ± 0·4 | 359 ± 7 | 2·5 ± 0·1 | 193 ± 2 |
| γ-Resorcylate | 2 | 6·8 ± 0·2 | 15·5 ± 0·4 | 257 ± 3 | 11·0 ± 0·1 | 20·1 ± 0·7 | 397 ± 4 | 5·1 ± 0·1 | 184 ± 2 |
| | 5 | 6·1 ± 0·1 | 14·6 ± 0·4 | 252 ± 3 | 8·9 ± 0·1 | 14·9 ± 0·7 | 357 ± 7 | 4·2 ± 0·1 | 191 ± 3 |
| | 10 | 5·1 ± 0·2 | 11·9 ± 0·2 | 205 ± 4 | 6·8 ± 0·1 | 10·6 ± 0·6 | 301 ± 4 | 3·5 ± 0·1 | 191 ± 2 |
| | 20 | 4·1 ± 0·1 | 7·9 ± 0·3 | 136 ± 4 | 4·9 ± 0·1 | 6·5 ± 0·2 | 235 ± 4 | 2·8 ± 0·1 | 193 ± 3 |

Each observation represents the mean of eight estimations ± S.D. Radioactivity is expressed as dis/min × $10^{-3}$.

overall glucose catabolism may be estimated by calculating the percentage of [1-$^{14}$C] glucose converted to $^{14}CO_2$. Since the radioactivity is lost from the [1-$^{14}$C] glucose as $^{14}CO_2$ at an early stage in the pentose phosphate pathway any radioactive lactate present must have been formed via glycolysis.

The results for the total glucose utilization and total lactate production (Table 3) show that salicylate and γ-resorcylate decreased the overall metabolism of glucose in human red cells, the effects being more pronounced with increasing concentrations of the drugs. The formation of radioactive lactate also progressively diminished, showing that salicylate and γ-resorcylate inhibited glycolysis under these experimental conditions. The reduced oxygen consumption and evolution of $^{14}CO_2$ showed that the drugs also interfered with the metabolism of glucose via the pentose phosphate pathway. Calculation of the percentages of [1-$^{14}$C] glucose converted to $^{14}CO_2$ and of the specific activities of the lactate formed indicated that the drugs inhibited glucose metabolism via the pentose phosphate pathway to a greater extent than by the glycolytic pathway.

One site of action of salicylate and γ-resorcylate on the pentose phosphate pathway must be on one or more of the enzyme systems responsible for the production of $^{14}CO_2$ from the [1-$^{14}$C] glucose. The results in Table 2 showed that both drugs inhibited hexokinase activity but the proportionally greater effects of salicylate and γ-resorcylate on the pentose phosphate pathway (Table 3) showed that an additional inhibitory effect must be present. The results in Table 4 show that all concentrations of salicylate and γ-resorcylate inhibited glucose-6-phosphate dehydrogenase activity in red cell haemolysates but that only the high concentrations (10 and 20 mM) of γ-resorcylate affected 6-phosphogluconate dehydrogenase activity.

## DISCUSSION

The results of the present work show that salicylate and γ-resorcylate cause multiple effects on the metabolism of glucose by human red cells. The patterns of distribution of radioactivity from [$^{14}$C] glucose into the soluble metabolic intermediates of red cell suspensions (Table 1) were almost entirely concerned with glycolytic intermediates. Salicylate and γ-resorcylate caused changes, the accumulation of radioactive glucose and pyruvate, which suggested that the drugs interfered with hexokinase and lactate dehydrogenase activities. Further work on haemolysed preparations (Table 2) showed that both enzyme activities were inhibited by the drugs *in vitro*. Salicylate and γ-resorcylate, in concentrations similar to those used in the present work, have been reported to inhibit lactate dehydrogenase activity in other biological systems.[16] However, little information is available about the effect of salicylate and γ-resorcylate on hexokinase activity except that low concentrations of salicylate (mM) did not inhibit the purified enzyme.[17]

γ-Resorcylate (10 and 20 mM), but not salicylate, caused an accumulation of labelled hexose monophosphates (Table 1) suggesting that it interfered with the further metabolism of these compounds. The results (Table 2) showed that γ-resorcylate inhibited phosphofructokinase activity in haemolysates and this site of action could explain the alterations found in the radioactive patterns.

The results of the experiments with the red cells metabolising glucose containing a trace of [1-$^{14}$C] glucose (Table 3) confirmed that both drugs affected the catabolism of glucose by the glycolytic pathway and in addition revealed that they interfered with

the pentose phosphate pathway. This latter route of glucose metabolism appeared to be more sensitive to the inhibitory actions of salicylate and γ-resorcylate than glycolysis. This result indicated that a site of inhibition of the pentose phosphate pathway other than an initial interference with hexokinase, which is common to both pathways, must be present. The results with haemolysates (Table 4) showed that the drugs inhibited glucose-6-phosphate dehydrogenase at all the concentrations used whereas 6-phosphogluconate dehydrogenase was only affected by the high concentrations of γ-resorcylate.

TABLE 4. EFFECTS OF SALICYLATE AND γ-RESORCYLATE ON DEHYDROGENASE ENZYME ACTIVITIES IN RED CELL HAEMOLYSATES

| Dehydrogenase | Salicylate (mM) | | | | γ-Resorcylate (mM) | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 | 5 | 10 | 20 | 2 | 5 | 10 | 20 |
| Glucose-6-phosphate | 8 | 12 | 19 | 33 | 6 | 12 | 31 | 37 |
| 6-Phosphogluconate | — | — | — | — | — | — | 5 | 15 |

Results are expressed as percentage inhibitions of enzyme activity and represent the mean of four estimations.

It is possible that the effects *in vitro* of salicylate found in the present work may have implications *in vivo*. In acute salicylate poisoning, plasma salicylate concentrations up to 10 mM (140 mg/100 ml) may be encountered and such concentrations could affect glucose metabolism in the circulating erythrocytes. During the therapy of rheumatic conditions, such as acute rheumatic fever, plasma salicylate concentrations of between 2 and 3 mM are attained and maintained for long periods. Such concentrations may interfere with lactate dehydrogenase activity and possibly glucose-6-phosphate dehydrogenase in the red cells.

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