MULTIPLE EFFECTS OF γ-RESORCYLIC ACID ON INTERMEDIARY METABOLISM

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Received August 11, 1961

γ-Resorcylic acid inhibited glutamic-pyruvic and glutamic-oxaloacetic transaminase activities in extracts of rat liver, kidney, brain and heart. Its effects on the distribution of radioactivity from [3-^{14}C] pyruvate among the soluble metabolic intermediates of chopped preparations of the rat tissues were consistent with this interference with transaminase enzymes. However, when [^{14}C] glucose and [1,4-^{14}C] succinate were incubated with isolated mitochondria and with a soluble fraction prepared from rat liver, the resorcylic acid produced marked changes in the incorporation of the radiocarbon into malic acid and fumaric acids, into an oligosaccharide fraction and into various phosphate compounds concerned in glycolytic reactions.

THE modes of action of antirheumatic drugs are obscure. Relatively little is known about the mechanisms by which such chemically diverse substances as steroids, salicylates and antimalarials produce their beneficial effects on the inflammatory processes which form a major part of the reaction of the body in rheumatic disease. One method of approach is to study the effects of the drugs on cellular metabolism and to attempt the correlation of any defined biological action with their anti-inflammatory properties. As a necessary preliminary the metabolic actions of the anti-rheumatic drugs must be discovered and explored. γ-Resorcylic acid (2,6-dihydroxybenzoic acid) has been reported to be an effective agent in the treatment of rheumatic fever (Reid, Watson, Cochran and Sproull, 1951). Its most pronounced biochemical action is an inhibition of rat serum glutamic-pyruvic transaminase activity *in vitro* (Steggle, Huggins and Smith, 1961). The present paper is concerned with a more detailed investigation of this property with particular reference to the effects of the drug on tissue metabolism. The inhibitory activity against the enzyme has been shown to occur in extracts of various rat tissues. The observed effects of resorcylic acid on the distribution of radioactivity from labelled pyruvate among the soluble metabolic intermediates of chopped preparations of the rat tissues were consistent with the hypothesis that the drug primarily affects transaminase enzymes. However, when labelled glucose and succinate were used in conjugation with rat liver preparations such as homogenates, mitochondria and a soluble fraction, it became evident that resorcylic acid produced multiple effects on intermediary metabolism.

EXPERIMENTAL

Tissue Preparations

Adult rats (wt. 200–250 g.) of the Wistar strain, maintained on M.R.C. cube diet No. 41, were killed by cervical fracture. Chopped suspensions

of liver, kidney, brain and heart muscle were prepared according to the directions of Smith and Moses (1960). Liver homogenates were prepared in 0·25M sucrose (5 g. wet weight of liver in 5 ml. sucrose solution) using a Potter-Elvehjem homogeniser with a Teflon pestle. Liver mitochondria, separated from 4 g. wet weight of liver by the method of Schneider and Hogeboom (1950), were suspended in 1 ml. of 0·25M sucrose. A soluble fraction from liver was prepared by centrifuging a 1 : 5 homogenate in 0·25M sucrose for 30 min. at 105,000 *g* in a Spinco preparative ultracentrifuge and discarding the pellet.

Radioactive Experiments

A solution of radioactive substrate (5μc in 5μl.) was added to 200μl. samples which contained 1–2 mg. dry weight of the tissue preparation. Resorcylic acid solution (10μl.) was added to produce a final concentration of 5 mM and an equivalent quantity of the appropriate incubation medium was added in the corresponding control experiments. The radioactive substrates, [^{14}C] glucose (72·90μc/μmole); sodium [3-^{14}C] pyruvate (3·62μc/μmole) and [1,4^{14}C] succinic acid (10·80μc/μmole) were obtained from The Radiochemical Centre, Amersham, Bucks. The chopped preparations were incubated for 3 hr. at 37° in the medium of Hastings, Teng, Nesbett and Sinex (1952) and the homogenates and subcellular fractions for 30 min. at 37° in 0·01M phosphate buffer containing (m-mole/l.): KCl, 10; $MgSO_4.7H_2O$,2; cytochrome C, 0·03 and ATP, 1. All the incubation media, with the exception of that used for the mitochondria, contained 10 mM glucose. At the end of the incubation period the tissue preparations were killed by the addition of 200μl. of boiling ethanol. The radioactively labelled intermediates were extracted, separated by two-dimensional paper chromatography, visualised by radio-autography and the ^{14}C measured by the techniques described previously (Smith and Moses, 1960).

Measurement of Transaminase Activities

The tissues (liver, kidney, brain or heart muscle) were homogenised in a Waring blendor for 3 min. in 2–3 volumes of 0·01M phosphate buffer at pH 7·6. After centrifuging for 60 min. at 11,000 *g* the supernatant was separated, dialysed for 18 hr. against 0·01M phosphate buffer and the pH readjusted to 7·6. Glutamic-pyruvic and glutamic-oxaloacetic transaminase activities were measured in the dialysates by the method of Reitman and Frankel (1957).

Results

The results in Table I show that 5 mM resorcylic acid significantly inhibited glutamic-pyruvic transaminase activity in extracts from rat liver, kidney, brain and heart muscle. Although the inhibition of the glutamic-oxaloacetic activity in the tissue extracts was less pronounced, all the tissue preparations showed significant effects with the drug.

The percentages of radiocarbon from [3-^{14}C] pyruvate which were incorporated into the soluble metabolic intermediates of the four tissues

in the presence or the absence of resorcylic acid are given in Table II. In the absence of the drug the qualitative patterns of incorporation of the isotope were similar for all the tissues. Most of the radioactivity was found in alanine and lactic acid which were derived from the pyruvate

TABLE I

INHIBITION OF GLUTAMIC-PYRUVIC AND GLUTAMIC-OXALOACETIC TRANSAMINASE ACTIVITIES IN RAT SERUM BY 5 mM γ-RESORCYLIC ACID

The results which are expressed as mean inhibitions per cent (± S.E.M.) have been analysed by the *t*-test and values of P are included. The minimum acceptable level of significance has been taken as P = 0·02. The number of observations are given in brackets.

Tissue	Glutamic-Pyruvic	P	Glutamic-oxaloacetic	P
Liver	81·5 ± 3·1 (8)	0·001	6·8 ± 0·6 (6)	0·001
Kidney	42·4 ± 4·1 (8)	0·001	22·7 ± 3·2 (5)	0·01
Brain	63·3 ± 1·3 (8)	0·001	9·9 ± 1·9 (6)	0·01
Heart	17·7 ± 0·8 (8)	0·001	11·5 ± 2·0 (6)	0·01

itself by transamination and dehydrogenation reactions respectively. A considerable proportion of the isotope was also incorporated into the amino-acids, glutamic and aspartic, which were formed from their corresponding α-keto acids (α-ketoglutaric and oxaloacetic) by transamination. The occurrence of radiocarbon in these compounds, as well as in citric and malic acids, is evidence that the pyruvate carbons entered

TABLE II

METABOLISM OF [3-^{14}C] PYRUVATE BY ISOLATED RAT TISSUES IN THE PRESENCE OR THE ABSENCE OF 5 mM γ-RESORCYLIC ACID

Results expressed as the total per cent ^{14}C incorporated from the labelled substrate into the sum of all the separated soluble intermediates; the ^{14}C in the residual substrate being excluded

Soluble Intermediate	Liver		Kidney		Brain		Heart	
	None	Resorcylate	None	Resorcylate	None	Resorcylate	None	Resorcylate
Alanine	61	31	30	25	18	9	51	32
γ-Aminobutyric acid	0	0	1	0	9	5	0	0
Aspartic acid	3	8	9	11	6	5	3	2
Glutamic acid	9	24	30	33	38	42	6	7
Citric acid	1	1	0	0	7	5	10	12
Lactic acid	25	33	25	29	14	28	19	37
Malic acid	1	0·5	4	3	3	2	6	6
Phosphates	1	2	1	2	0·4	1	2	2
Unidentified compounds	0·3	2	0	0	4	3	2	2

the tricarboxylic acid cycle. The distribution of the radioactivity from the labelled pyruvate among the soluble intermediates is illustrated in the radioautogram (Fig. 1).

The major effect produced by resorcylic acid was a substantial reduction in the formation of labelled alanine accompanied by increased incorporations of radioactivity into the other fractions.

The results in Table III show the distribution of radioactivity from labelled succinate among the soluble intermediates of various preparations of rat liver. These comprise a chopped preparation, a homogenate and a

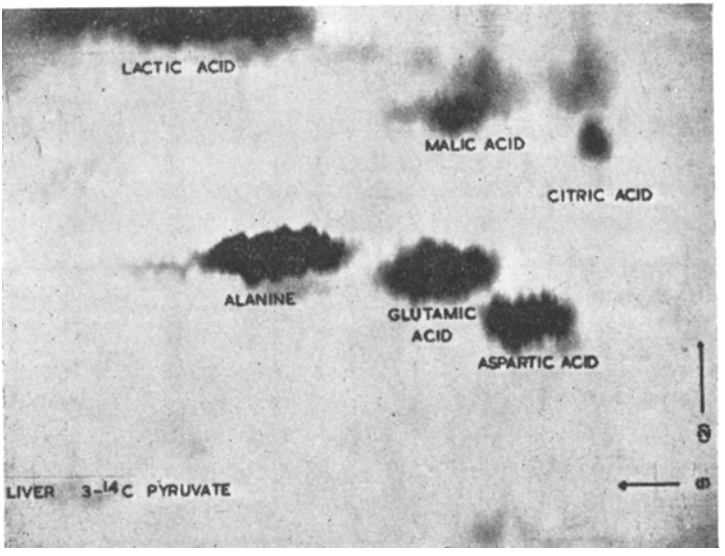


FIG. 1. Radioautogram of paper chromatogram showing distribution of radioactivity from [3-^{14}C] pyruvate among the soluble metabolic intermediates of a chopped preparation of rat liver. In this, and the subsequent figures, the solvent systems used for chromatography are as follows: (1) phenol-water; (2) butanol-propionic acid-water. The chromatograms represent extracts prepared from one-fifth of the reaction mixture. Exposed 14–21 days. U.K. represents unidentified compounds.

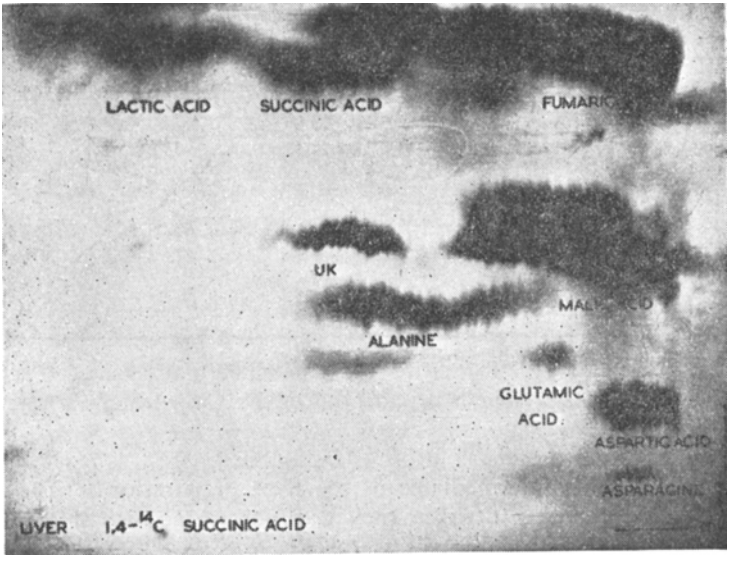


FIG. 2. Distribution of radioactivity from [1,4-^{14}C] succinate among the soluble metabolic intermediates of a chopped prepation of rat liver.

suspension of mitochondria. The radioautogram (Fig. 2) shows the distribution of ^{14}C in the chopped preparation. The soluble fraction prepared from rat liver did not metabolise the labelled succinate. In the control experiments, malic and fumaric acids were the compounds which contained the major proportion of the radioactivity with smaller amounts

TABLE III

METABOLISM OF [1,4-^{14}C] SUCCINATE BY RAT LIVER PREPARATIONS IN THE PRESENCE OR THE ABSENCE OF 5 mM γ-RESORCYLIC ACID

(Results expressed as in Table II)

Soluble Intermediate	Chopped preparation		Homogenate		Mitochondria	
	None	Resorcylate	None	Resorcylate	None	Resorcylate
Alanine	1	0·3	8	4	1	0·5
Aspartic acid	2	1	20	17	25	7
Asparagine	0·1	0	0	0	5	0
Glutamic acid	0·1	0·2	8	6	2	1
Glutamine	0	0	1	0	19	0
Citric acid	0	0	0	0	1	4
Fumaric acid	22	22	15	23	5	21
Lactic acid	0·4	1	3	6	16	1
Malic acid	74	75	34	42	7	63
Phosphates	0	0	0	0	12	0
Unidentified compounds	0·3	0	1	1	5	0

being found in aspartic acid, glutamic acid, alanine and lactic acid. A consistent effect of the resorcylic acid in all the preparations was a reduction of the incorporation of ^{14}C into the amino-acids (alanine, aspartic and glutamic). However, the most prominent action of the drug was concerned with the tricarboxylic cycle acids in the mitochondrial

TABLE IV

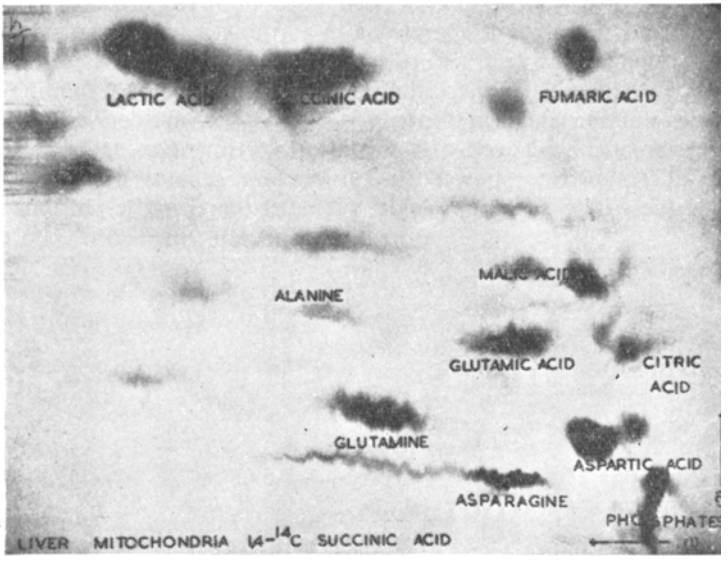
METABOLISM OF [^{14}C] GLUCOSE BY RAT LIVER PREPARATIONS IN THE PRESENCE OR THE ABSENCE OF 5 mM γ-RESORCYLIC ACID

(Results expressed as in Table II)

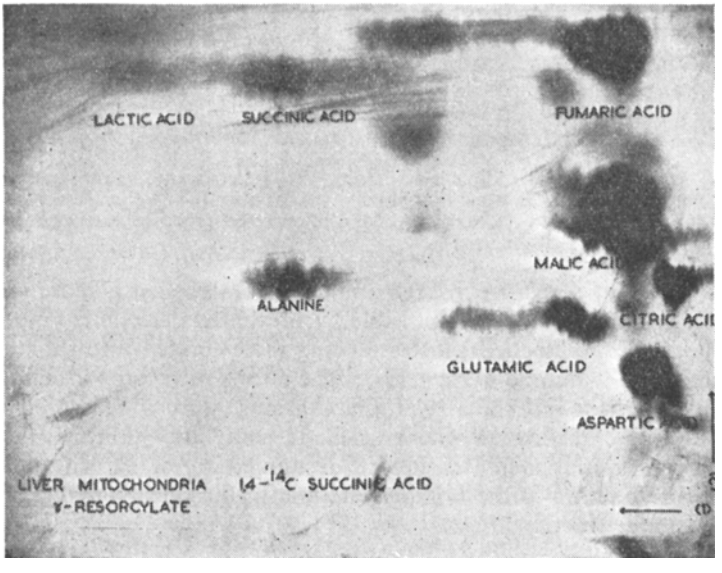
Soluble Intermediate	Chopped preparation		Homogenate		Soluble fraction	
	None	Resorcylate	None	Resorcylate	None	Resorcylate
Alanine	9	7	13	7	16	10
γ-Aminobutyric acid	14	9	0	0	1	1
Aspartic acid	14	16	0	0	16	13
Glutamic acid	14	14	0	0	0·5	0·3
Glutamine	0	0	0	0	0·5	0
Oligosaccharides	0	0	36	67	0	0
Citric acid	1	1	0	0	0	0
Lactic acid	7	5	14	0	37	26
Malic acid	0	0	0	0	1	1
Phosphates	30	32	37	16	24	48
Unidentified compounds	9	16	0	9	1	2

experiments. Here, the formation of the labelled malic acid and fumaric acid were substantially increased and this effect is illustrated in the radioautograms (Fig. 3).

Table IV shows the distribution of radioactivity from labelled glucose among the soluble intermediates of the liver preparations. The mitochondria are excluded because they did not metabolise this labelled substrate. In the absence of resorcylate, the ^{14}C was incorporated mainly into various phosphate compounds, which are intermediates in glycolysis, into lactic acid and into the amino-acids, alanine, aspartic acid,



(a)



(b)

FIG. 3. Incorporation of ^{14}C from [1,4–^{14}C] succinate into metabolic intermediates of a mitochondrial suspension from rat liver. (a) control; (b) in the presence of 5 mM γ-resorcylic acid.

glutamic acid and γ-aminobutyric acid. The radioautogram (Fig. 4) was made from the chopped preparation of liver. The liver homogenate differed from the other preparations in forming a high proportion of a labelled oligosaccharide fraction, which on acid hydrolysis yielded only radioactive glucose, and in the virtual absence of the amino-acids (aspartic, glutamic and γ-aminobutyric acids) which are derived by way of the tricarboxylic acid cycle. A separate experiment using [^{14}C] glucose as the only substrate showed that liver homogenates were capable of forming these labelled amino-acids. It must therefore be presumed that

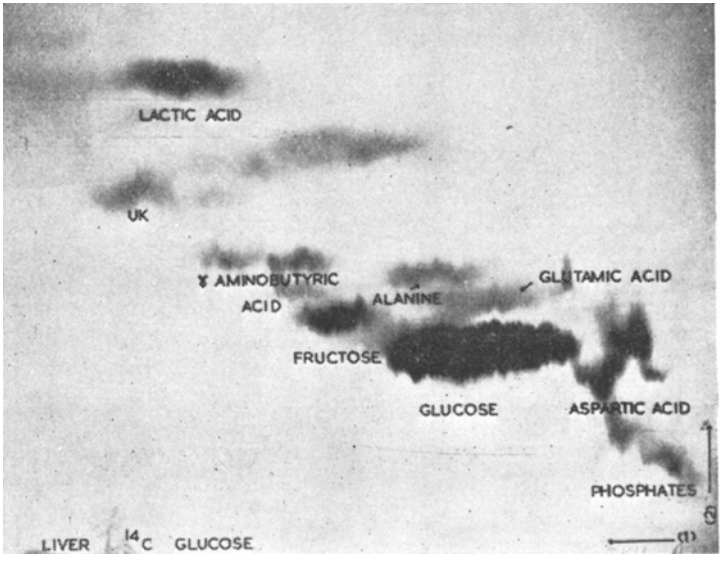


FIG. 4. Distribution of radioactivity from [^{14}C] glucose among the soluble metabolic intermediates of a chopped preparation of rat liver. The glucose spot contains about 90 per cent of the ^{14}C activity of the paper; the fructose was a contaminant of the original labelled glucose.

the presence of a relatively large amount of unlabelled glucose (10 mM) in the experiments recorded in Table IV prevented detectable amounts of isotope from the labelled substrate being incorporated into the aspartic, glutamic and γ-aminobutyric acids. The effects of resorcylic acid, which were common to all three liver preparations, were a reduction of the formation of labelled alanine and lactic acid. In addition, the drug caused substantial increases in the accumulation of ^{14}C in the oligosaccharide fraction in the homogenate and in the phosphate compounds in the soluble fraction.

DISCUSSION

The results show that the inhibitory activity of resorcylic acid against rat serum glutamic-puruvic transaminase is also evident in extracts prepared from isolated rat tissues. The drug also inhibited glutamic-oxaloacetic transaminase activity in the extracts although to a smaller

extent. The interference with glutamic-pyruvic transaminase activity is reflected in the altered distribution of radioactivity from labelled pyruvate among the soluble metabolic intermediates of the isolated tissues. Thus, resorcylic acid caused a substantial reduction in the formation of radioactive alanine in the chopped preparations of rat liver, kidney, brain and heart. The diminished conversion of the labelled pyruvate to alanine caused a higher degree of incorporation of radiocarbon into substances, such as lactic acid, which are formed from pyruvate carbons by metabolic pathways insensitive to the action of resorcylate. A similar, but smaller reduction in the incorporation of radioactivity into alanine also occurred in the liver preparations incubated either with the labelled glucose or with the labelled succinate in the presence of the resorcylic acid. In the mitochondrial suspensions incubated with the radioactive succinate, the resorcylic acid caused a decreased incorporation of the isotope into aspartic acid which suggested that the drug also inhibited the glutamic-oxaloacetic transaminase in this particular experimental system.

In the liver preparations the resorcylic acid produced several effects which appeared to be distinct from its inhibition of transaminase enzymes. The most striking action, illustrated in Fig. 3, was a large increase in the incorporation of ^{14}C into the malic and fumaric acid fractions of rat liver mitochondria incubated with labelled succinate. These changes may reflect corresponding increases in the pool sizes of the acids due to an inhibition of malic dehydrogenase. Alternatively, the resorcylic acid may have altered the permeability of the mitochondrial membranes causing an increased escape of the tricarboxylic cycle acids formed initially inside the subcellular particles.

A further effect of the drug was concerned with the liver homogenate supplied with labelled glucose (Table IV). This preparation converted a large proportion of the incorporated radioactivity into an oligosaccharide fraction probably through the mediation of hepatic maltotransglucosylase (cf. Stetten and Stetten, 1960). This enzyme is capable of forming a series of malto-oligosaccharides from glucose and suitable acceptors, for example maltose and maltotriose. The relatively large amount of labelled oligosaccharide found in the liver homogenate may be due to the availability of these acceptor substances formed during glycogen breakdown in this preparation. In the chopped liver preparation which was incubated for a much longer period, 3 hr. as opposed to 30 min. for the homogenate, the oligosaccharide acceptor substances may have been further degraded to smaller molecules and in the soluble fraction from liver they were probably removed during the preparative procedures. The effect of resorcylic acid in almost doubling the extent of incorporation of radioactivity from the labelled glucose into the oligosaccharide fraction could follow either from a direct action on an enzyme system including the maltotransglucosylase or more indirectly by increasing the amounts of suitable acceptor substances, such as maltotriose, in the homogenate.

In the soluble fraction of the liver a large fraction of the ^{14}C was incorporated into phosphate compounds formed during glycolysis. The presence of resorcylic acid increased the proportion of radiocarbon into

the phosphates suggesting that the drug may have inhibited a reaction concerned with the glycolytic breakdown of the labelled substrate.

Acknowledgements. We are grateful to Mr. W. Smith for preparing the photographs of the radioautograms, to the Empire Rheumatism Council and the Medical Research Council for grants towards the cost of the work and to the Wellcome Trust for a grant for the purchase of a Spinco preparative ultracentrifuge.

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