Biochemical Pharmacology, 1965, Vol. 14, pp. 1701–1706. Pergamon Press Ltd., Printed in Great Britain.

INFLUENCE OF RIFAMYCINS ON GLYCOLYSIS OF RAT BRAIN

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(*Received* 25 *March* 1965; *accepted* 13 *July* 1965)

Abstract—Rifamycin S and rifamycin SV are the oxidized and reduced form of a reversible oxido-reduction system, whereas rifamycin B-diethylamide can no longer be oxido-reduced. Both rifamycin SV and B-diethylamide stimulate lactate output of rat brain preparations endowed with glycolytic activity; rifamycin SV only stimulates pyruvate output by brain homogenate. The presence of glycolyzing supernatant as well as mitchondria of brain homogenate, aerobiosis and addition of NAD are required for rifamycins effects to occur. Rifamycins do not inhibit the activity of a lactate dehydrogenase preparation of brain *in vitro*. The data suggest that rifamycins exert their activity mainly on phosphate turnover of brain homogenate; rifamycin SV seems to exert an additional effect on respiratory activity of brain mitochondria.

A CHROMOPHORE naphtoquinone group has been identified in the structure of the antibiotics belonging to the rifamycins.[1] The grouping is in the reduced (hydroquinone) state in rifamycin SV, and can undergo oxidation to quinone, rifamycin SV being oxidized to rifamycin S.[2] Rifamycin SV and S differ in their absorption spectra, with maxima at 445 mμ and 525 mμ at pH 7·3 respectively.[3] Rifamycins have been prepared, in which the naphto hydroquinone group can no longer undergo oxidation: in rifamycin B-diethylamide the group is blocked by esterification.[2] Owing to their structure, rifamycins could be expected to exert some influence on oxidations of animal tissues, especially on the respiratory chain localized in mitochondria. An inhibitory effect was shown to be exerted by rifamycin SV on oxidative phosphorylation by liver, heart and brain mitochondria.[4] In previous experiments, rifamycin SV was observed to stimulate aerobic glycolysis, as measured by lactate production, of slices of rat liver, diaphragm or brain, incubated in the presence of glucose.[4] In further investigations, rifamycin SV as well as rifamycin B-diethylamide were shown to increase lactate output by brain homogenate, whereas an increase in pyruvate output by the same homogenate was observed in presence of rifamycin SV only;[4, 5] the enhancing activity of rifamycin SV on lactate occurred provided both supernatant and mitochondrial fractions of brain homogenate were present in the incubation mixture.[5]

The present report is concerned with a more detailed investigation on the effects of rifamycin SV and rifamycin B-diethylamide on aerobic glycolysis of brain *in vitro*. Experiments have been performed on tissue slices, tissue homogenates and also, on single enzymatic activities.

METHODS

Male and female rats of the Wistar strain, body weight 250–350 g, were used throughout.

Slices of liver or brain cortex were cut with razor blades according to Deutsch[6] and held in cooled Krebs Ringer phosphate solution until used. Livers and brains were

homogenized in 9 vol. of ice-cold sucrose 0·25 M or sucrose 0·25 + EDTA 0·001 M in a Potter-Elvehjem-type homogenizer. Homogenates were centrifuged in the cold at 1085 *g* for 5 min, supernatants were used as such or fractionated.

Glycolysis was studied with sucrose + EDTA homogenate, adenosine-triphosphatase (ATPase) with mitochondria isolated from sucrose homogenate.

Sucrose-EDTA brain homogenate was fractionated by centrifuging in the cold at 20,200 *g* for 15 min in a supernatant fraction, composed of soluble supernatant and microsomes, and a mitochondrial fraction, composed mostly of mitochondria. Supernatant was recentrifuged at the same speed. Mitochondria were washed and centrifuged twice at 20,200 *g* for 15 min and finally suspended in sucrose-EDTA to 200 mg equivalents of fresh tissue/0·1 ml. Protein content of liver homogenate was 10·4 ± 0·6 (D.S.) mg/ml; of brain homogenate was 5·3 ± 0·4 mg/ml. Protein content of brain supernatant fraction was 4·9 ± 0·3 mg/ml; of brain mitochondrial fraction was 7·6 ± 1·3 mg/ml.

Sucrose homogenates were centrifuged in the cold at 20,200 *g* for 15 min, mitochondria washed and centrifuged twice, and suspended in sucrose to a protein content of 1·7 ± 0·2 (D.S.) mg/ml and 1·6 ± 0·1 mg/ml for liver and brain mitochondria respectively. Protein was estimated by micro-kjeldahl procedure.

A crude lactate dehydrogenase preparation was obtained from rat brain following the method of Kornberg for muscle.[7] Brains were homogenized in potassium hydrate 0·03 M at 4°, the homogenate centrifuged at 12,000 *g* for 10 min, the supernatant precipitated by addition of solid ammonium sulphate to 0·9 saturation. The precipitated paste was dissolved in water, insoluble material removed by centrifugation, ammonium sulphate saturated at 2° was added to 0·52 saturation and the solution exposed to 10° for 30 min. The precipitate collected by centrifugation was dissolved in water and reprecipitated at the same saturation. The resulting bottom layer was dissolved in water; specific activity (μmoles NADH oxidized/min/mg protein)[7] was about 40.

Lactate was estimated by the method of Barker and Summerson,[8] pyruvate by the method of Koepsell and Sharpe.[9] Phosphoric esters in the incubation mixture were fractionated as barium salts, according to Umbreit *et al.*,[10] inorganic phosphate was estimated by the method of Fiske and Subbarow.[11] Phosphoric esters were also checked by paper chromatography separation. The following solvents were chosen: 80 ml tert. butanol/20 ml water + 4 g picric acid; 90 ml tert. amyl alcohol/90 ml water/30 ml formic acid 90%.[12] Phosphorylated intermediates were detected by the reagent of Hanes and Isherwood.[12]

Adenosine triphosphatase activity of mitochondria was assayed in presence of EDTA and in absence of Mg, as described by Recknagel and Anthony.[13] Nucleotides and substrates were obtained from commercial sources. Compositions of reaction mixtures and conditions of incubation are described in legends accompanying the tables.

RESULTS

Data on aerobic glycolysis of liver and brain slices are reported in Table 1. Brain produced more lactate in the presence than in the absence of rifamycin SV, whereas the drug did not affect lactate output by liver slices. In other incubation experiments, almost the same amount of pyruvate was observed to be released by brain slices in the presence of either rifamycin SV or rifamycin B-diethylamide.

TABLE 1. EFFECT OF RIFAMYCIN SV ON AEROBIC GLYCOLYSIS OF RAT LIVER AND BRAIN SLICES

Compound added	Lactate produced (μmoles/100 mg dry wt)	
	Liver	Brain
—	14·2 ± 1·7	38·7 ± 5·6
Rifamycin SV $1·3 \times 10^{-3}$ M	15·1 ± 0·8	64·8 ± 9·7

Slices, 150 mg, were incubated in Krebs Ringer phosphate, pH 7·4, with glucose 0·032 M, total volume 3 ml, 60 min at 37° in O_2 atmosphere. Incubation was stopped by 0·15 ml trichloroacetic acid 100%, mixture was centrifuged, lactate was estimated in supernatant. Data are the mean value ±S.D. of 5 experiments.

Results of Table 2 demonstrate that the addition of rifamycins to brain homogenate increased lactate output, an increase of pyruvate having been observed by addition of rifamycin SV only. There were no significant changes by liver homogenate.

Table 3 shows that both mitochondrial and supernatant fractions had to be added to the incubation medium for the enhancing effect of rifamycins on glycolysis to occur. Rifamycin SV inhibited slightly lactate output by supernatant alone, whereas rifamycin B-diethylamide failed to affect it significantly. When mitochondria were added in excess equivalents to supernatant in the incubation mixture, lactate output diminished by 60%. Further addition of rifamycins blocked the lowering effect of mitochondria on lactate output, rifamycin B-diethylamide having been more effective than rifamycin SV in that respect. Rifamycin SV, added to the incubation mixture supplied with both mitochondria and supernatant, induced an increase in pyruvate output much higher than that of supernatant alone.

TABLE 2. EFFECTS OF RIFAMYCIN SV AND RIFAMYCIN B-DIETHYLAMIDE ON LACTATE AND PYRUVATE OUTPUT OF RAT LIVER AND BRAIN HOMOGENATE.

Compound added	Liver		Brain	
	lactate	pyruvate	lactate	pyruvate
—	2·8 ± 2·1[a]	0·11 ± 0·08	10·8 ± 2·7	0·16 ± 0·01
Rifamycin SV 10^{-3} M	1·6 ± 1·3[b]	0·14 ± 0·02	43·3 ± 8·9	2·57 ± 0·47
Rifamycin B-diethylamide 10^{-3} M	1·3 ± 0·7[c]	0·10 ± 0·01	42·2 ± 4·5	0·19 ± 0·05

Data are given in μmoles in incubation mixture/hr. The homogenate (1 ml) was incubated with phosphate buffer pH 7·4 45 μmoles, KCl 75 μmoles, $MgSO_4 + 7H_2O$ 20 μmoles, nicotinamide 24 μmoles, glucose 30 μmoles, ATP, AMP, NAD 3 μmoles each, total volume 3 ml, for 60 min at 37° in O_2 atmosphere. Incubation was stopped by 0·15 ml trichloroacetic acid 100%. Protein content see text.

Data are the mean value ±S.D. of 6 experiments.

Significance: P = 0·4–0·3 for a to b; 0·3–0·2 for a to c.

TABLE 3. INFLUENCE OF RIFAMYCINS ON LACTATE AND PYRUVATE OUTPUT BY RAT BRAIN HOMOGENATE FRACTIONS

Compound added	Homogenate fractions		
	Mitochondrial	Supernatant	Mitochondrial + Supernatant
		Lactate	
—	5·5 ± 0·8	29·6 ± 4·7[a]	11·7 ± 2·6[b]
Rifamycin SV 10^{-3} M	3·6 ± 0·9	21·9 ± 5·1[c]	28·3 ± 7·0[d]
Rifamycin B-diethylamide 10^{-3} M	6·7 ± 1·3	31·7 ± 4·0[e]	36·9 ± 1·6[f]
		Pyruvate	
—	0·06 ± 0·02	0·33 ± 0·07	0·14 ± 0·04
Rifamycin SV 10^{-3} M	0·15 ± 0·01	0·23 ± 0·04	2·20 ± 0·26
Rifamycin B-diethylamide 10^{-3} M	0·04 ± 0·09	0·23 ± 0·04	0·18 ± 0·09

Data are given in μmoles in incubation mixture/hr. 1 ml mitochondrial fraction + 1 ml sucrose-EDTA, 1 ml supernatant fraction + 1 ml sucrose-EDTA, or 1 ml mitochondrial fraction + 1 ml supernatant fraction were added to the reaction mixture, as composed as in Table 2, in 3 ml total volume. Conditions of incubation as in Table 2. Protein content see text.

Data are the mean value ± S.D. of 6 experiments.

Significance: $P < 0{\cdot}001$ for a to b, b to d, b to f; $P = 0{\cdot}01$–$0{\cdot}001$ for a to c; $P = 0{\cdot}02$–$0{\cdot}01$ for e to f; $P = 0{\cdot}05$–$0{\cdot}02$ for c to d.

It has been reported that rifamycins did not influence glycolysis by incubation in anaerobiosis.[5] Experiments have been performed to examine the effects of withdrawing single incubation mixture components on rifamycins activity; NAD was requisite for rifamycins effects to take place.

In previous experiments on oxidative phosphorylation, rifamycin SV at 10^{-3} M concentration was shown to inhibit inorganic phosphate uptake by phosphorylating mitochondria by 100%, whereas rifamycin B-diethylamide inhibited at the same concentration by 50%.[4] Phosphoric esters from the incubation mixture, analyzed by barium fractionation, presented significant variations only on acid-easily hydrolyzable

TABLE 4. EFFECT OF RIFAMYCINS ON PHOSPHATE ESTERS OF RAT BRAIN INCUBATE

Compound added	Acid-labile P, Ba-insoluble fraction	Alkali-labile P, ethanol-insoluble fraction
—	8·7 ± 1·4	2·6 ± 0·6
Rifamycin SV 10^{-3} M	4·6 ± 0·9	1·4 ± 0·2
Rifamycin B-diethylamide 10^{-3} M	4·6 ± 0·8	0·8 ± 0·1

Data are given in μmoles inorganic phosphorus (P) estimated after hydrolysis of the related fraction of the incubation mixture/hr.

Homogenate was composed by 1 ml mitochondrial fraction + 1 ml supernatant fraction in 3 ml total volume of incubation mixture. Composition of reaction mixture and conditions of incubation: see Tables 2 and 3.

Data are the mean value ± S.D. of 4 experiments.

(7 min at 100° in HCl N) phosphate of the barium insoluble fraction, and on alkali-hydrolyzable (20 min at 25° in NaOH 2N) phosphate of the ethanol insoluble fraction. Acid-labile phosphate, corresponding to adenine nucleotides di- and tri-phosphates and to fructose-diphosphate, diminished with either rifamycins. In addition, adenine nucleotides appeared to be decreased by paper chromatography. Alkali-labile phosphates, corresponding to triose-phosphates, diminished also, the major decrease having been observed with rifamycin B-diethyalmide. These results are summarized in Table 4.

TABLE 5. INFLUENCE OF RIFAMYCINS ON LACTATE-DEHYDROGENASE OF RAT BRAIN

Compound added	μmoles pyruvate /10 min	μmoles lactate /10 min
—	0·86	3·80
Rifamycin SV 10^{-3} M	0·91	3·95
Rifamycin B-diethylamide 10^{-3} M	0·86	3·89

Incubation mixture: phosphate buffer pH 7·4 50 μmoles, K pyruvate 5·1 μmoles, NADH 6 μmoles, crude lactate dehydrogenase 100 μg protein, total volume 5 ml. Incubation was stopped after 10 min at 37° with 0·5 ml TCA 100%, lactate and pyruvate estimated in incubate. Data are the mean value of 2 experiment.

Table 5 shows that rifamycins did not influence the brain lactate dehydrogenase activity *in vitro*. Table 6 reports the data on the stimulating effect of rifamycins on ATP-ase activity of mitochondria of liver and brain.

In view of the results on the influence of rifamycins on mitochondria, it seemed worth while to test the influence of 2,4-dinitrophenol 10^{-4} and 5×10^{-4} M on glycolysis of reconstructed brain homogenates. 2,4-dinitrophenol was shown to behave like rifamycin SV in increasing lactate and pyruvate output.

TABLE 6. STIMULATION OF ATPASE OF RAT LIVER AND BRAIN MITOCHONDRIA BY RIFAMYCINS

Compound added	Inorganic phosphate from ATP	
	Liver mitochondria	Brain mitochondria
—	0·6 ± 0·02	1·3 ± 0·1
Rifamycin SV 10^{-3} M	1·2 ± 0·04	1·5 ± 0·1
Rifamycin B-diethylamide 10^{-3} M	1·1 ± 0·02	1·8 ± 0·1

Data are given in μmoles in incubation mixture/15 min.

Reaction mixture: ATP K salt 6 μmoles, Tris-HCl buffer pH 7·6 20 μmoles, EDTA 2 μmoles, mitochondria 1 ml in 2 ml total volume, incubated 15 min at 30°. Reaction stopped with 0·1 ml trichloroacetic acid 100%, inorganic phosphate estimated in supernatant after centrifugation.

Protein content: see text.

Data are the mean value ±D.S. of 4 experiments.

DISCUSSION

Both rifamycin SV or B-diethylamide show an influence on brain glycolysis *in vitro* in intact cells and in homogenate. Both supernatant and mitochondrial fractions must be present in reconstructed homogenate. Supernatant of brain homogenate is endowed with glycolytic activity; respiring mitochondria added in excess to brain supernatant inhibit glycolysis, a Pasteur effect *in vitro* resulting from the interaction between the two fractions.[14, 15, 16] The Pasteur effect, measured as lactate output, is inhibited by rifamycins, rifamycin B-diethylamide being more active than rifamycin SV. The drugs exert their effect probably at the level of phosphate turnover,[17] as is demonstrated by phosphorylated intermediates analysis, influence on mitochondrial ATP-ase, and absence of inhibition on lactate dehydrogenase. Rifamycin SV only, on the other hand, increases pyruvate output. This fact is probably related with the oxido-reductive state of supernatant fraction,[18] on which rifamycin SV, owing to the reduced state of the naphtoquinone nucleus, could exert some influence, not a direct one, though mediated through mitochondria.

Rifamycin SV inhibits phosphorylative oxidation of mitochondria,[4] and 2,4-dinitrophenol showed a similar effect to that of rifamycin SV on glycolysis of brain homogenate.

Experiments are in progress to get a better insight into the co-ordination mechanism between glycolysis and respiration on brain homogenate, and into the influence of rifamycins on mitochondrial activities.

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