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EFFECTS OF DICOUMAROL, INSULIN AND ANOXIA ON RAT DIAPHRAGM—I. EFFECT ON RESPIRATION, EXTRACELLULAR SPACE AND WATER CONTENT

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Abstract—Dicoumarol (0·5 mM) inhibits the respiration of muscle tissue, but at a low concentration (0·05 mM) enhances respiration. The effect of insulin when given with dicoumarol, when acting as a stimulus, is one of enhancement.

WE HAVE previously reported[1] that dicoumarol stimulates insulin release. In order to throw light on the mechanism by which this effect is produced we considered it of interest to find out if dicoumarol had any effect on the absorption of glucose. We observed that dicoumarol produced a remarkable decrease in glucose uptake by the striated muscle. In agreement with these results a decrease of the intestinal adsorption of this sugar has also been reported.[2] As these results were not in agreement with those found by other authors,[3] who used different uncouplers of oxidative phosphorylation, we carried out a study of the effect of dicoumarol on the passage of some sugars into the striated muscle. We express the amount of sugar as a "space", the sugar "space", comparing this "space" with the extracellular fluid of the tissue. In this paper we present the results obtained from the extracellular fluid (determined by measuring the sorbitol space) with two different preparations of rat diaphragm.

METHODS AND MATERIALS

Incubation medium. All the incubations were carried out in Krebs–Ringer II buffer in a Dubnoff incubator with agitation. The medium was maintained at 37° and continuously gassed with $O_2 + CO_2$ (95:5%) or N_2 (100%). Sugars were added at the concentrations stated in the tables.

Animals. Diaphragm muscle was obtained from male and female albino Wistar rats of 100–130 g body wt, fed on our stock laboratory diet. Food was withheld from the intact rats for 18 hr before the experiments. The animals had free access to water at all times.

Cut diaphragm preparation. Rats were killed by decapitation and exsanguinated. Diaphragms were then immediately removed, and spread on filter paper previously soaked in the incubation buffer. The posterior segment was removed and the remainder divided along the central tendon to yield two hemi-diaphragms. Each one

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of these hemi-diaphragms was divided into two pieces. Each flask of incubation contained two pieces of about 50 mg each, from different hemidiaphragms in 2 ml of incubation medium.

Intact diaphragm preparation. Rats were killed by decapitation and exsanguinated. The intact diaphragm was obtained as described by Kipnis and Cori.[4] After washing, each diaphragm was incubated in the incubation flask containing 10 ml of incubation medium.[1]

Chemicals. D-Glucose was obtained from Merck; D-sorbitol from British Drug Houses; D-xylose and vitamin K_3 from Sigma; dicoumarol from Mann, R.L.; [U-^{14}C]-D-sorbitol and [U-^{14}C]-D-xylose were from the Radiochemical Centre, Amersham. [^{14}C]-Dicoumarol from Junta de Energía Nuclear, Madrid.

Crystalline insulin (Lilly) was dissolved in HCl N/300 at a concentration of 10 units/ml, 0·4 mg. This stock solution was diluted with the buffer to the required concentration just before each experiment.

Analytical methods. Analyses of sugars from the muscle were made on extracts prepared by boiling the muscle in 2 ml of water for 15 min and deproteinizing with $Zn(OH)_2$.[5] All the sugars were completely extracted by this procedure. Samples of medium after incubation were suitably diluted and deproteinized by this procedure.

D-Xylose was estimated photometrically by the *p*-bromoaniline method.[6] D-Glucose was also estimated photometrically by the Somogyi[5] modification of Nelson's method.[7] For assays of [^{14}C]-D-sorbitol, [^{14}C]-D-xylose and [^{14}C]-dicoumarol, samples, already deproteinized, were pipetted onto crystal or aluminium planchets, dried under an infrared lamp and counted in a gasflow counter.

Total water was determined by weighing the muscle before and after drying at 110°.

Calculations. All the calculations were obtained from those of Randle and Smith.[3,8]

Statistical analysis. The significance of the differences between means has been established by caclulating *t*. P, the probability of differences due to chance, was obtained from tables for *t*.[9]

RESULTS

Effect of dicoumarol, insulin and vitamin K_3 (menadione) on the respiration of rat diaphragm. Results already published[8] showed that some uncouplers of oxidative phosphorylation have an effect on the respiration of the tissue. This fact prompted us to find out if dicoumarol had any effect on the respiration of rat cut diaphragm and, if so, to compare its effect with that of insulin[10] and vitamin K_3, an antagonist of dicoumarol in oxido reduction processes.

As shown in Figs. 1 and 2, low concentrations of dicoumarol (0·05 mM) stimulate the respiration of the tissue (oxygen uptake) whereas high concentrations (0·5 mM) inhibit respiration. The inhibition by dicoumarol is not reversed by insulin. Nevertheless vitamin K_3 which has a slight effect by itself, partially reversed the inhibition produced by dicoumarol, Fig. 3. Figures 4 and 5 show the results obtained on the respiration of the tissue in the absence of substrate (glucose). These results are very similar to those previously stated.

Total water content of intact diaphragm. Table 1 shows that insulin does not produce any variation in the total water content, whereas dicoumarol slightly increases it. Our

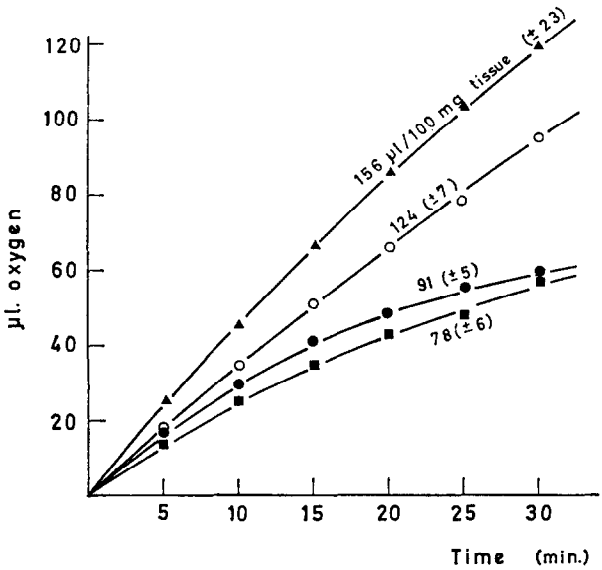

FIG. 1. Effect of dicoumarol and insulin on the respiration of rat cut diaphragm. Incubation medium: Krebs–Ringer II, glucose (3 mg/ml). Incubation time, 40 min; stabilization time, 10 min. ○——○, Control; ▲——▲, insulin (0·1 units/ml); ■——■, dicoumarol (0·5 mM); ●——●, insulin (0·1 units/ml + dicoumarol (0·5 mM). (○ ▲ ■ ●) Mean of six experiments. Each value represents the total oxygen consumption in 40 min of incubation ± S. E. M.

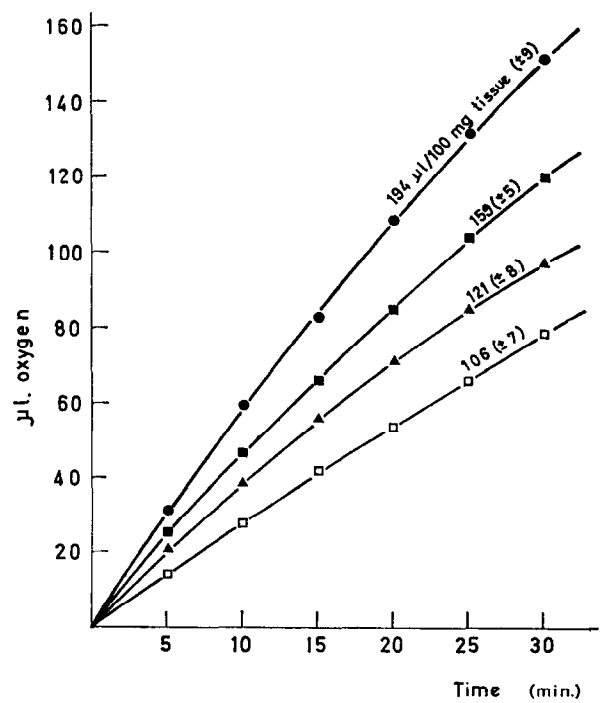

FIG. 2. Effect of dicoumarol and insulin on the respiration of rat cut diaphragm. Incubation medium, Krebs–Ringer II, D-glucose (3 mg/ml). Incubation time, 40 min; stabilization time, 10 min. □——□, Control; ▲——▲, insulin (0·1 units/ml); ■——■, dicoumarol (0·05 mM); ●——●, insulin (0·1 units/ml) + dicoumarol (0·05 mM). (□ ▲ ■ ●) Mean of eight experiments. Each value represents the total oxygen consumption in 40 min of incubation ± S. E. M.

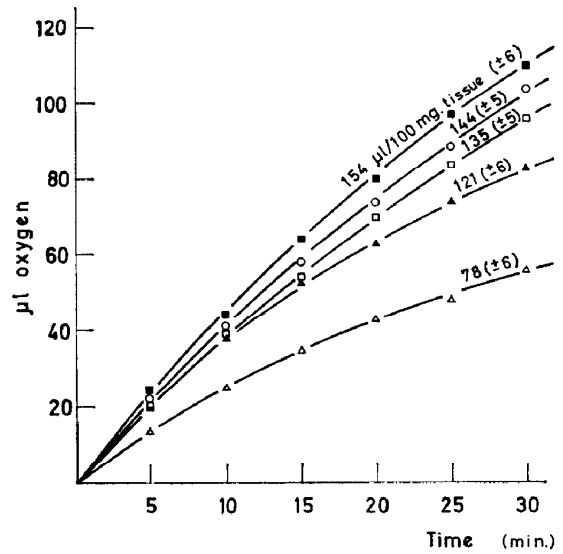

FIG. 3. Effect of dicoumarol and Vitamin K_3 on the respiration of rat cut diaphragm. Incubation medium, Krebs–Ringer II, D-glucose (3 mg/ml). Incubation time, 40 min; stabilization time, 10 min. ○——○, Control; □——□, sodium bisulphite (0·5 mM); △——△, dicoumarol (0·5 mM); ■——■, vitamin K_3 (bisulphite salt, 0·5 mM); ▲——▲, vitamin K_3 + dicoumarol (both, 0·5 mM). (○ □ △ ■ ▲) Mean of ten experiments. Each value represents the total consumption in 40 min of incubation ± S. E. M.

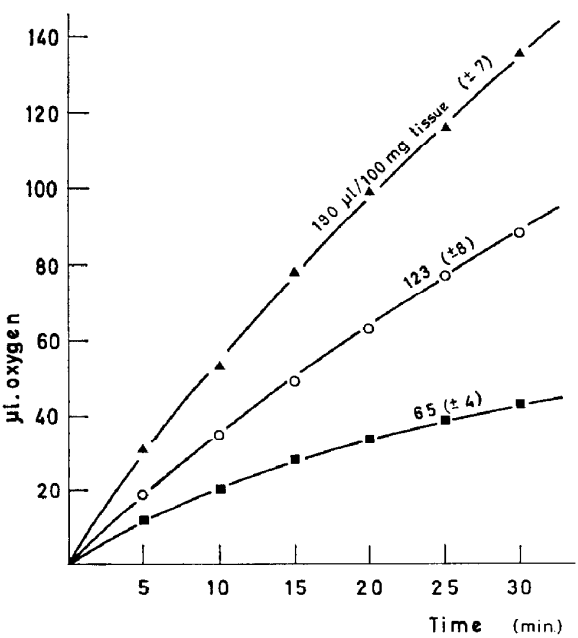

FIG. 4. Effect of dicoumarol on the respiration of rat cut diaphragm. No substrate added. Incubation medium, Krebs–Ringer II. Incubation time, 40 min; stabilization time, 10 min. ○——○, Control; ■——■, dicoumarol (0·5 mM); ▲——▲, dicoumarol (0·05 mM). (○ ▲ ■) Mean of six experiments. Each value represents the total oxygen consumption in 40 min of incubation ± S. E. M.

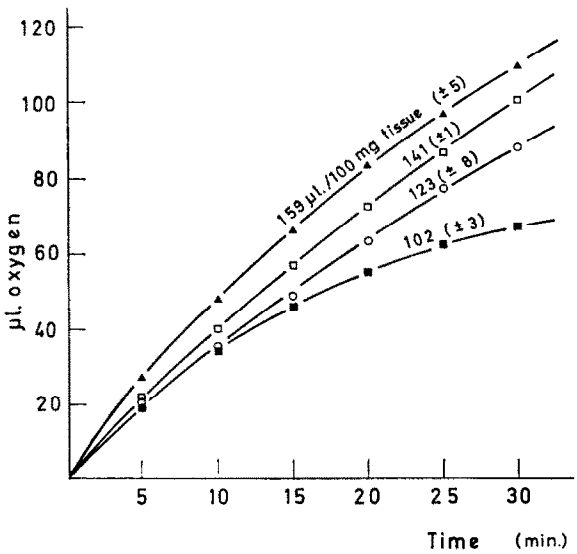

FIG. 5. Effect of insulin and dicoumarol on the respiration of rat cut diaphragm. No substrate added. Incubation medium, Krebs–Ringer II. Incubation time, 40 min; stabilization time, 10 min. ○——○, Control; □——□, insulin (0·1 units/ml); ▲——▲, insulin (0·1 units/ml) + dicoumarol (0·05 mM); ■——■, insulin (0·1 units/ml) + dicoumarol (0·5 mM). (○ □ ▲ ■) Mean of ten experiments. Each value represents the total oxygen consumption in 40 min of incubation ± S. E. M.

values (81:7%) are slightly higher than those described by Randle: 70%[8] and Kipnis: 78%.[4]

Effect of dicoumarol on the extracellular space of rat diaphragm. As two different preparations of rat diaphragm (cut and intact diaphragm) had to be used we carried out a preliminary study of the extracellular fluid in both preparations and we investigated whether dicoumarol had any effect on either parameter.

Table 2 shows the effect of dicoumarol on the extracellular space (sorbitol space). As can be seen from this table, the value of the extracellular space does not remain constant during the incubation time, which suggests that the cellular membrane might be damaged. Dicoumarol strongly increases the sorbitol space. We observed,

TABLE 1. EFFECT OF INSULIN AND DICOUMAROL ON THE TOTAL WATER CONTENT OF INTACT DIAPHRAGM

Addition	No. exp.	Total water (ml/100 g of wet tissue)	Difference ± S.E.M. of the difference with control	Significance of difference (P)
None (control)	22	81·73		
Insulin 0·1 units/ml	22	81·69	0·04 ± 0·24	N.S.
Dicoumarol 0·5 mM	22	83·54	1·81 ± 0·20	P < 0·01
Insulin 0·1 units/ml + dicoumarol 0·5 mM	22	83·94	2·21 ± 0·26	P < 0·01

Incubation time: 60 min, incubation medium, Krebs–Ringer II. N.S., not significant.

TABLE 2. COMPARISON BETWEEN SORBITOL AND DICOUMAROL SPACES OF RAT CUT DIAPHRAGM

Incubation time (min)	Dicoumarol (mM)	Sorbitol space (ml/100 g of diaphragm) Mean ± S. E. M.	Significance of the difference with control (*)	Glucose uptake (mg/g of wet diaphragm/hr of incubation) Mean ± S. E. M.
60		60·9 ± 1·6 (8)*		2·52 ± 0·15 (7)
60	0·5	76·3 ± 1·7 (8)	P < 0·01	1·29 ± 0·22 (5)
120		76·4 ± 1·7 (5)	P < 0·02	4·10 ± 0·18 (3)
120	0·5	107·0 ± 2·6 (5)	P < 0·01	2·31 ± 1·18 (3)
		Dicoumarol space		
60	0·5	216·9 ± 20·9 (28)	P < 0·01	1·29 ± 0·07 (15)

Sorbitol space, Krebs–Ringer II, D-glucose (3 mg/ml), D-sorbitol (0·12 mg/ml), [^{14}C]-D-sorbitol (0·05 μCi/ml).

Incubation medium dicoumarol space, Krebs–Ringer II, D-glucose (3 mg/ml), D-sorbitol (0·12 mg/ml), [^{14}C]-dicoumarol (0·05 μCi/ml).

Number of observations are given in parentheses. D-Glucose analyses were by the colorimetric method of Somogyi.

when using [^{14}C]-dicoumarol, that the value of dicoumarol space is higher than that of sorbitol space, we therefore assume that the drug penetrates into the cell.

Effect of dicoumarol on the extracellular space of the intact rat diaphragm. The effect of dicoumarol on the respiration of rat diaphragm could not be understood if the drug did not penetrate into the cell. From data shown in Table 2 it may be assumed that the drug penetrated the cell, however, it was necessary to reproduce these results with an intact diaphragm.

TABLE 3. COMPARISON BETWEEN SORBITOL AND DICOUMAROL SPACES OF RAT INTACT DIAPHRAGM

Incubation time (min)	Dicoumarol (mM)	Sorbitol space (ml/100 g of wet diaphragm) Mean ± S. E. M.	Dicoumarol space (ml/100 g of wet diaphragm) Mean ± S. E. M.	Significance of the difference betwen both spaces (P)
60	0·05	43·2 ± 2·8 (7)	38·9 ± 1·7 (16)	N.S.
60	0·5	41·2 ± 1·4 (9)	59·2 ± 3·2 (5)	P < 0·02
120	0·5	55·8 ± 3·7 (4)	113·4 ± 6·7 (5)	P < 0·05

Sorbitol space, Krebs–Ringer II, D-sorbitol (0·12 mg/ml), D-xylose (3·5 mg/ml), [^{14}C]-D-sorbitol (0·05 μCi/ml).

Incubation medium dicoumarol space, Krebs–Ringer II, D-sorbitol (0·12 mg/ml), D-xylose (3·5 mg/ml), [^{14}C]-dicoumarol (0·05 μCi/ml).

Number of observations are given in parentheses. N.S., not significant.

Table 3 shows that dicoumarol concentrations of 0·05 and 0·5 mM produce no variation in the extracellular fluid after 60 min of incubation, but produce a slight increase after incubation of 120 min. On the other hand, we compared the values of the sorbitol and [^{14}C]-dicoumarol spaces in respect to two variants: (a) drug concentration in the medium and, (b) incubation time. The values obtained with a concentration of dicoumarol (0·05 mM) with 120 min incubation time proved that the drug had penetrated into the cell.

TABLE 4. EFFECT OF ANOXIA ON THE EXTRACELLULAR SPACE OF RAT INTACT DIAPHRAGM

	Sorbitol space (ml/100 g of wet diaphragm)	Significance of the difference (P)	Xylose space (ml/100 g of wet diaphragm)	Significance of the difference (P)
Aerobic control	43·0 ± 1·1 (8)	N.S.	46·8 ± 1·5 (8)	P = 0·01
Anaerobic control	39·7 ± 0·9 (14)		66·0 ± 2·8 (14)	

Incubation medium, Krebs–Ringer II, D-xylose (3·5 mg/ml), D-sorbitol (0·12 mg/ml), [^{14}C]-D-sorbitol (0·05 μCi/ml).
Incubation time: 60 min.
Number of observations given in parentheses. D-xylose analyses were by the colorimetric method of Roe and Rice.

Effect of anoxia on the extracellular space of intact rat diaphragm. As may be seen in Table 4, anoxia does not modify the extracellular space of the tissue and increases the accumulation of D-xylose into the cell.

DISCUSSION

The results presented in this study are in agreement with those of other authors,[8] in relation to the effect of different uncouplers of oxidative phosphorylation on the respiration of the tissue. All these compounds show the double and opposite effect that we obtained by varying the concentrations used.

Most of the proposed mechanisms for the incubation of mitochondrial respiration by uncouplers of oxidative phosphorylation have attempted to explain both the uncoupling and the inhibition by a common reaction. Harris *et al.*[11] suggest that the uncoupling of mitochondrial respiration inhibits K^+ transport, which is required for substrate transport into the mitochondria. Dam and Slater[12] suggest that uncouplers act as anions, competing with substrate anions for entry into the mitochondria via an energy-requiring transport system. As no differences between the results with or without substrate were observed, we found ourselves in agreement with Wilson and Merz,[13] in that there is no direct relationship between the uncoupling and the inhibition, and that the inhibition has a direct effect on the suitable dehydrogenase(s).

According to Krebs, insulin produces an increase of the respiration which is due to a direct effect of the hormone on the citric-acid cycle. The fact that insulin is not able to revert the inhibition produced by dicoumarol suggests that the hormone and the uncoupler do not act on the same system and that dicoumarol, as an inhibitor, is more powerful than insulin as a stimulus.

Martius and Nitz-Litzow,[14] working with mitochondria from the liver of deficient hens, and Brodie and Ballantine[15] with the bacterial system, reached the same conclusion: *viz.*, that restoration of oxidation and phosphorylation is specifically dependent upon the addition of vitamin K_1 or upon a closely related homologue. Compounds containing a methyl group in the C-2 position and an unsaturated side chain of at least 5 carbon atoms in the C-3 position of the naphthoquinone ring are active in restoring both activities. Restoration of oxidation alone occurs with a number of naphthoquinones substituted in the C-2 or C-3 position of vitamin K_3.

With the K-reactivated system, as with untreated extracts, oxidation can be uncoupled from phosphorylation by low concentrations of 2–4 dinitrophenol, dicoumarol, lapachol, thyroxine and triiodothyroacetic acid. Lapachol and dicoumarol were found to be competitive inhibitors of phosphorylation at low concentrations. With higher concentrations of dicoumarol a noncompetitive or mixed type of inhibition of oxidation was observed; however, the inhibition of phosphorylation remained competitive.

There appears to be a large degree of agreement between these results and those which we obtained. High concentrations of dicoumarol inhibit the respiration of rat diaphragm and vitamin K_3 partially reverts this effect, possibly competing with it, in oxide-reduction reactions.

Several authors have studied the uptake of dicoumarol by different tissues. Howland[16] found that dicoumarol entered mitochondria rapidly, approaching maximum uptake in about 10 sec at 0°. Wosilait[17] showed that the amount of dicoumarol uptake by rat liver slices is proportional to the amount of the drug added to the medium, and reached a maximum in about 30 min. Dicoumarol was bound by the soluble, mitochondrial, microsomal and nuclear fractions of liver in a specific way.

In comparison with these results, we conclude that the uptake of dicoumarol by rat diaphragm is also proportional to the concentration of the drug in the medium, although penetration was slower than in the previously stated systems, since the saturation level in our system required an incubation period of 120 min.

From this it can be seen that dicoumarol penetrates into the cell and therefore the drug may act on the systems responsible for phosphorylation and oxidation.

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REFERENCES

1. M. A. GUNTHER, C. L. QUIJADA and J. L. R-CANDELA, *Life Sci.* **5**, 1091 (1966).
2. F. PONZ and M. LLUCH, *Revista Española de Fisiologia* **24**, 203 (1968).
3. P. J. RANDLE and G. H. SMITH, *Biochem. J.* **70**, 490 (1958).
4. D. M. KIPNIS and C. F. CORI, *J. biol. Chem.* **224**, 681 (1957).
5. M. SOMOGYI, *J. biol. Chem.* **160**, 69 (1945).
6. J. H. ROE and R. W. RICE, *J. biol. Chem.* **173**, 507 (1948).
7. M. NELSON, *J. biol. Chem.* **153**, 507 (1948).
8. P. J. RANDLE and H. G. SMITH, *Biochem. J.* **70**, 501 (1958).
9. J. S. DE LA LOMA, *Experimentación Agrícola*. Hispano-American, México, D.F. (1955).
10. H. A. KREBS and L. V. EGGLESTON, *Biochem. J.* **32**, 913 (1938).
11. E. J. HARRIS, M. P. HOFER and B. C. PRESSMAN, *Biochemistry* **6**, 1348 (1967).
12. K. VAN ADAM and E. C. SLATER, *Proc. natn. Acad. Sci. U.S.A.* **58**, 2015 (1967).
13. D. F. WILSON and R. D. MERZ, *Archs Biochem. Biophys.* **119**, 470 (1967).
14. C. MARTIUS and D. NITZ-LITZOW, *Biochim. biophys. Acta* **12**, 134 (1953).
15. A. F. BRODIE and J. BALLANTINES, *J. biol. Chem.* **235**, 226 (1960).
16. J. L. HOWLAND, *Biochem. J.* **106**, 317 (1968).
17. W. D. WOSILATT, *Biochem. Pharmac.* **17**, 429 (1968).