THE EFFECT OF NORADRENALINE AND ADRENOCHROME ON CARBOHYDRATE METABOLISM OF RAT DIAPHRAGM

by

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As shown by the authors[1] in the CORIS' laboratory, adrenalin einhibits glucose uptake in the isolated rat diaphragm. In an extension of this work the problem has been considered whether the metabolic effects of adrenaline are due to the adrenaline molecule as such or to conversion products of adrenaline. Thus, a study has been made of the effects of "protected" adrenaline, adrenochrome and noradrenaline on carbohydrate metabolism in muscle.

It is known that noradrenaline affects the carbohydrate metabolism in a manner similar to that of adrenaline both *in vivo* (SAHYUN[2], BEARN, BILLING AND SHERLOCK[3]) in the perfused liver (HOUSSAY AND GERSHMAN[4]) and *in vitro* in liver slices (SUTHERLAND[5]) and in the isolated rat diaphragm (WALAAS AND WALAAS[6]). It is characteristic that the metabolic effect of noradrenaline is considerably weaker than that of adrenaline. In the present investigation a comparison between the effect of noradrenaline and adrenaline on the isolated rat diaphragm has been made.

It is more uncertain whether adrenochrome has any physiological effect on carbohydrate metabolism. MURACCIOLE AND RUIZ-GIJON[7] thus found that adrenochrome gave no definite hyperglycemic reaction after injection in rabbits. However, it was demonstrated that adrenochrome inhibits the glycolysis in homogenates and extracts of rat brain (RANDALL[8], MEYERHOF AND RANDALL[9]). In the present paper it is shown that adrenochrome inhibits hexokinase activity in extracts of the rat diaphragm. Other data indicate that rat diaphragm hexokinase requires free sulfhydryl groups for its activity.

EXPERIMENTAL

Young male rats weighing 100–140 g were employed. The diaphragms were incubated in Krebs-Ringer phosphate medium, pH 7.4; 140 mg glucose added per 100 ml medium. The experimental conditions have previously been described in detail (WALAAS AND WALAAS[1]). In the experiments *d,l*-noradrenaline and *l*-noradrenaline bitartrate monohydrate have been used. Both these compounds were pure, prepared by Professor U. S. VON EULER and kindly placed at our disposal*.

Pure adrenochrome was prepared by oxidation of adrenaline with silver oxide (MCCARTHY[10])*.

Glucose was determined by NELSON's method[12], glycogen according to WALAAS AND WALAAS[1] and lactic acid after the method of BARKER AND SUMMERSON[13].

Extracts of diaphragm hexokinase were made by pooling 1 g diaphragm tissue from 6 rats. The diaphragms were removed and kept in ice-cold Krebs-Ringer medium. Thereupon they were blotted, weighed, cut into small pieces and homogenized in a Potter-Elvehjem all-glass grinder

* The purity of the compounds was confirmed by spectrophotometric examinations according to the method of LUND[11].

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for 10 min. 10% homogenate in 0.25 *M* sucrose + 0.01 *M* Tris (hydroxymethyl) aminomethane buffer (Tris), pH 8.0, was prepared. All manipulations were carried out at + 1° C. The homogenate was centrifuged at 8000 × *g* for 20 min in an "International" Refrigerated Centrifuge and the supernatant used.

0.5 ml of the extract was incubated in a reaction mixture containing (concentrations): 0.001 *M* glucose, 0.003 *M* ATP, 0.01 $MgCl_2$, 0.05 *M* KF, 0.03 *M* Tris buffer pH 8.2. Total volume 3.0 ml. Incubation for 20 min at 30° C in air. The reaction was stopped with 1.0 ml 0.3 *N* $ZnSO_4$ and 1.0 ml 0.3 *N* $Ba(OH)_2$. Glucose disappearance was determined in the filtrate by the method of NELSON[12]. Adrenochrome was converted to a stable product in the filtrate by standing overnight. Correction for its effect on the NELSON method was made. Ba-salt of ATP from "Sigma" chemical company was converted to K-salt before use.

Beckmann D.U. spectrophotometer was used for the spectrophotometric measurements.

RESULTS

Experiments on the isolated diaphragm

It appears from Table I that noradrenaline inhibits net glycogen synthesis as well as the uptake of glucose in the isolated rat diaphragm§. These are the same effects as previously shown for adrenaline. Fig. 1 indicates that the effect of *l*-noradrenaline on glycogen synthesis is weaker than that of *l*-adrenaline; *d,l*-noradrenaline has a considerably weaker effect. It is seen from Fig. 2 that a similar situation is shown

TABLE I

THE EFFECT OF NORADRENALINE ON THE GLUCOSE UPTAKE AND GLYCOGEN SYNTHESIS IN THE ISOLATED RAT DIAPHRAGM

Group	Glucose uptake		Glycogen synthesis
	mg/g/h		
Without *l*-noradrenaline	1.8	(3)	1.5 ± 0.15 (6) *
With $3 \cdot 10^{-5}$ *M* *l*-noradrenaline	1.1	(3)	0 ± 0.27 (6) *
Without *d,l*-noradrenaline	2.2 ± 0.13	(8) **	1.6 ± 0.24 (6) ***
With $3 \cdot 10^{-5}$ *M* *d,l*-noradrenaline	1.4 ± 0.13	(8) **	0.5 ± 0.28 (6) ***

Incubation in 1 ml medium with 140 mg glucose per 100 ml, for 60 min at 37° C, 100% O_2. Number of experiments in ( ). Statistical test on the significance of the differences:
* *t*: 4.8, <P: 0.001; ** *t*: 4.2, P: 0.001; *** *t*: 3.4, P: 0.004.

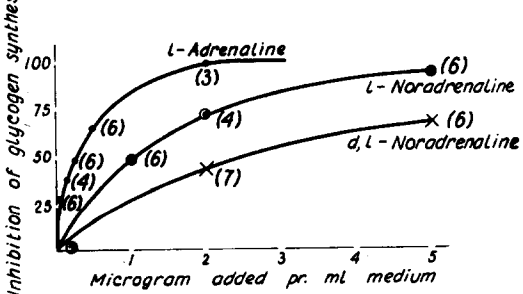

Fig. 1. The inhibition of glycogen synthesis in the isolated diaphragm by adrenaline and noradrenaline, expressed in per cent of maximum inhibition. Maximum inhibition: 1.6 mg/g/h = 100. Experimental conditions as given in Table I. Number of experiments in ( ).

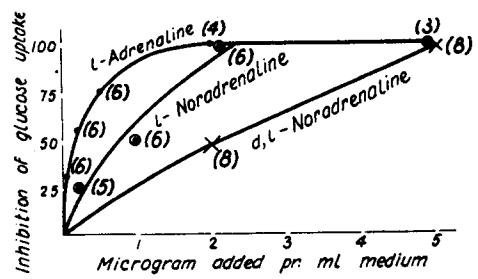

Fig. 2. The inhibition of glucose uptake in the isolated diaphragm by adrenaline and noradrenaline, expressed in per cent of maximum inhibition. Maximum inhibition: 0.8 mg/g/h = 100. Experimental conditions as given in Table I. Number of experiments in ( ).

§ It has also been shown in some experiments that addition of *l*-noradrenaline results in increased lactic acid production in the diaphragm.

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for the three compounds, regarding the inhibition of the glucose uptake. It appears further from Table II that in the isolated rat diaphragm *l*-adrenaline is 4 times more active than *l*-noradrenaline and 10 times more active than *d,l*-noradrenaline when the effects on glycogen synthesis and glucose uptake are concerned.

TABLE II

THE RELATIVE EFFECTS OF ADRENALINE AND NORADRENALINE ON THE GLUCOSE UPTAKE AND GLYCOGEN SYNTHESIS IN THE RAT DIAPHRAGM *in vitro*

(The values are calculated from Figs. 1 and 2.)

Compound	*Inhibition of glycogen synthesis*		*Inhibition of glucose uptake*	
	50% inhibition is given by:	*Relative activity*	*50% inhibition is given by:*	*Relative activity*
l-Adrenaline	0.25 μg	1	0.20 μg	1
l-Noradrenaline	1.0 μg	1/4	0.75 μg	1/4
d,l-Noradrenaline	2.5 μg	1/10	2.0 μg	1/10

In order to elucidate whether the metabolic effects of adrenaline are due to the molecule itself or to oxidation products, spectrophotometric investigations have been carried out after incubation. It is known that adrenaline is oxidized in neutral salt solutions (CHAIX, CHAUVET AND JEZEQUEL[14]) under aerobic conditions. Oxidation was arrested at the adrenochrome stage in bicarbonate buffer, while adrenochrome was oxidized further in phosphate buffer (CHAIX, MORIN AND JEZEQUEL[15]). Investigations reported in Fig. 3 show that adrenaline was quickly oxidized aerobically to adreno-

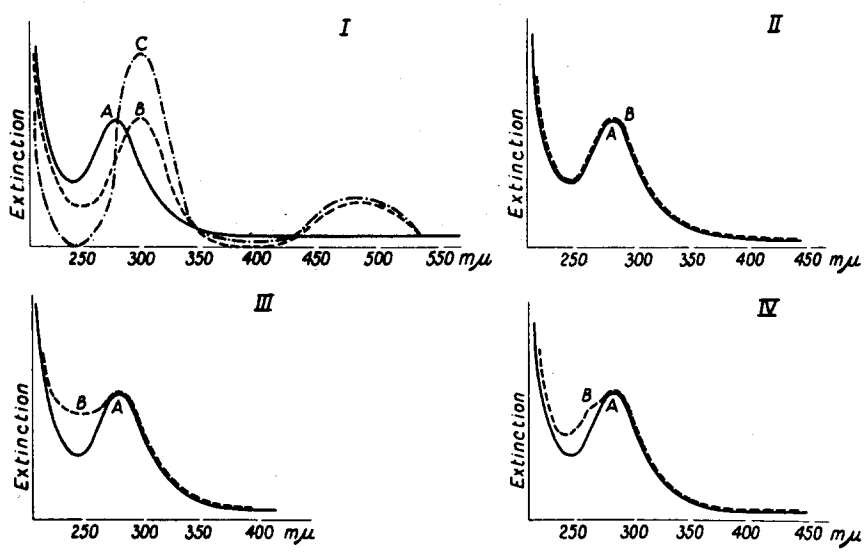

Fig. 3. Spectrophotometric examinations of the oxidation of adrenaline in Krebs-Ringer-phosphate-medium, pH 7.4, 140 mg glucose per 100 ml, at 37° C. I. *Aerobic conditions.* A. Before incubation. B. After incubation for 5 min, 100% O_2. C. After 30 min incubation, 100% O_2. II. *Anaerobic conditions.* A. Before incubation. B. After 30 min incubation, 100% N_2. III. *Adrenaline "protected" by glutathione.* A. Before incubation. B. After 30 min incubation in a medium which contained 0.002 *M* glutathione, 100% O_2. IV. *Diaphragm added during incubation.* A. Before incubation. B. After 60 min incubation, 100% O_2. 2 hemidiaphragms were added to 4 ml incubation medium.

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chrome in Krebs-Ringer phosphate medium at pH 7.4 and 37° C and oxidation was stopped at this stage. On the other hand, this conversion was inhibited by either anaerobiosis or the addition of glutathione. When the diaphragm was added to the incubation medium, oxidation of adrenaline was prevented even in oxygen. The observation therefore indicates that the metabolic effects on the diaphragm are due to unchanged adrenaline. A corresponding observation was made with noradrenaline.

This was further investigated in experiments in which glutathione was added to the incubation medium (Table III). The effect of adrenaline on the glycogen synthesis, glucose uptake and lactic acid production was here essentially the same as without the addition of glutathione. Finally an experiment was carried out in which adrenochrome was added *in vitro* to the incubation medium (Table IV). 2 μg adrenochrome per ml medium gave no metabolic effect on the diaphragm, and 10 μg per ml medium was also without any definite effect. The experiments thus give further indication that the metabolic effects on the diaphragm are caused by the unoxidized adrenaline.

TABLE III

THE EFFECT OF ADRENALINE PROTECTED BY GLUTATHIONE ON THE RAT DIAPHRAGM *in vitro*

Group	*Glucose uptake**	*Glycogen synthesis***	*Lactic acid production*
	mg/g/h		
Without adrenaline	2.6 ± 0.14 (9)	1.3 ± 0.25 (8)	0.6 ± 0.07 (4)
With adrenaline	1.8 ± 0.12 (9)	0.3 ± 0.27 (8)	0.75 ± 0.07 (4)

Incubation in 1 ml medium. Glutathione in a concentration of 0.002 *M* was present. 2 μg adrenaline was added.

Incubation for 60 min at 37° C, 100% O_2. Number of experiments in ( ).

Statistical test on the significance of the differences:

* *t*: 4.2, P: 0.001; ** *t*: 2.7, P: 0.02.

TABLE IV

THE EFFECT OF ADRENOCHROME ON RAT DIAPHRAGM *in vitro*

Group	*Glucose uptake*	*Glycogen synthesis*	*Lactic acid production*
	mg/g/h		
Without adrenochrome	2.7 ± 0.11 (9)	1.5 ± 0.14 (9)	0.7 ± 0.15 (6)
With $1.1 \cdot 10^{-5}$ *M* adrenochrome	2.8 ± 0.09 (9)	1.4 ± 0.11 (9)	0.7 ± 0.14 (6)
Without adrenochrome	2.2 ± 0.29 (4)	1.9 ± 0.18 (5)	
With $5.5 \cdot 10^{-5}$ *M* adrenochrome	1.9 ± 0.15 (4)	1.7 ± 0.11 (5)	

Incubation in 1 ml medium, for 60 min at 37° C, 100% O_2. Number of experiments in ( ).

Experiments on extracts of the diaphragm

Sulfhydryl agents. The assumption (RANDALL[8]) that adrenochrome inhibits glycolysis by oxidation of SH-groups has been tested. Indications have been obtained that diaphragm hexokinase require free sulfhydryl groups for activity. The enzyme thus resembles brain particle hexokinase (SOLS AND CRANE[16]). The hexokinase activity is gradually lost during incubation at 30° C. As shown in Table V the metal-binding

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agent versene preserves the activity of the enzyme. The thermal inactivation of the unprotected enzyme is irreversible and activity is not restored by versene.

TABLE V

PROTECTION OF DIAPHRAGM HEXOKINASE BY VERSENE

Preincubation (1)	*Addition before test of activity (2)*	*Residual activity (3) μM glucose utilized*
Versene	Water	1.97
Water	Water	0.80
Water	Versene	0.96
Not preincubated		1.98

0.5 ml of diaphragm extract was made up to 1.5 ml with neutral 0.0015 *M* versene (concentration). Tris buffer pH 8.0 to a final concentration of 0.03 *M* was included. The tubes were incubated for 1 hour at 30° C. After cooling, the additions as indicated in column 2 were made. The flasks were fortified with glucose, ATP, $MgCl_2$, KF and Tris buffer, pH 8.2, according to the standard procedure and incubated for 20 min at 30° C.

As reported in Table VI the SH-agent *p*-chloromercuribenzoate (PCMB) produced complete inhibition of hexokinase activity. With concentration of PCMB which produced partial inactivation, reversal of the activity was obtained by cysteine. In an experiment where diaphragm extract was preexposed to $2.7 \cdot 10^{-5} M$ PCMB for 3 min at 20° C an inhibition of 79% of hexokinase activity was observed. 0.001 *M* cysteine partially restored the activity when added during the final incubation, the inhibition then being 22%.

Experiments with *o*-iodosobenzoate likewise indicated the presence of essential sulfhydryl groups. An inhibition of 78% was observed when the extract was incubated with 0.0016 *M* *o*-iodosobenzoate prior to the measurement of hexokinase activity.

TABLE VI

INHIBITION OF RAT DIAPHRAGM HEXOKINASE BY *p*-CHLOROMERCURIBENZOATE

PCMB concentration	*Inhibition %*
$2.7 \cdot 10^{-4} M$	100
$2.7 \cdot 10^{-5} M$	66
$1.4 \cdot 10^{-5} M$	34
$5.4 \cdot 10^{-6} M$	0

0.5 ml of extract was mixed with PCMB at + 1° C. After 2 minutes the usual incubations mixture as given in the general procedure was added and the flasks were incubated at 30° C for 20 minutes. Glucose phosphorylation in control flask was 2.8 μ*M*.

TABLE VII

INHIBITION OF RAT DIAPHRAGM HEXOKINASE BY ADRENOCHROME

Adrenochrome concentration	*Inhibition %*
$1 \cdot 10^{-3} M$	40
$8 \cdot 10^{-4} M$	35
$5 \cdot 10^{-4} M$	21
$2 \cdot 10^{-4} M$	0

0.5 ml of extract was mixed with adrenochrome at + 1° C. After 2 minutes the usual incubation mixture was added and incubation was performed at 30° C for 20 minutes. Glucose phosphorylation in control flask was 2.4 μ*M*.

Adrenochrome. As shown in Table VII adrenochrome partially inhibits activity of diaphragm hexokinase in the extract*. The extent of inhibition was not increased when the extract was preexposed to adrenochrome for a prolonged time. The effect by adrenochrome was partially reversed by glutathione. In an experiment in which $8 \cdot 10^{-4} M$ adrenochrome exerted 50% inhibition the addition of 0.002 *M* glutathione reduced the inhibition by 50%. As described elsewhere (WALAAS[17]) hexokinase

* In other experiments the same results were obtained when 2-deoxy-D-glucose was used as the substrate instead of D-glucose. Thus, the inhibition of glucose phosphorylation by adrenochrome cannot be attributed to an effect on phosphofructokinase.

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activity in extracts of the diaphragm is only slightly inhibited by adrenaline while noradrenaline is without any effect.

Cysteine was not able to reverse the inhibition by adrenochrome. This may be explained by the fact that cysteine rapidly converts the red adrenochrome to a yellow fluorescent compound, which is resistant against reduction with sodium hydrosulfite. At pH 7.4 in phosphate buffer the compound has two absorption maxima at 390 mμ and 250 mμ. In its properties it resembles adrenolutine; it may however be an addition compound of adrenochrome with cysteine. Glutathione, when added in great excess, at pH 7.4 in phosphate buffer, converts adrenochrome to a colourless compound with an absorption maximum at 305 mμ. This is probably leuco-adrenochrome. By reduction of adrenochrome with $Na_2S_2O_4$ a colourless compound with 2 absorption maxima at 240 mμ and 350 mμ is formed.

The possibility that adrenochrome inhibits glycolysis by acting as a phosphate acceptor has also been considered (WAJZER[18,19]). This has not been supported by the experiments reported in Table VIII. The inhibition of hexokinase activity by adrenochrome was of the same order of magnitude at different concentrations of ATP. Similar results are reported by MEYERHOF AND RANDALL[9] in experiments on glycolysis in brain homogenate.

TABLE VIII

THE EFFECT OF ADRENOCHROME ON THE HEXOKINASE REACTION IN RAT DIAPHRAGM HOMOGENATE

Adrenochrome moles	*ATP moles*	*Glucose phosphorylation μg glucose*	*Inhibition*	
			μg glucose	*per cent*
0	$3 \cdot 10^{-3}$	371		
$5 \cdot 10^{-4}$	$3 \cdot 10^{-3}$	264	107	29
0	$1.5 \cdot 10^{-3}$	264		
$5 \cdot 10^{-4}$	$1.5 \cdot 10^{-3}$	138	108	44
0	$8 \cdot 10^{-4}$	128		
$5 \cdot 10^{-4}$	$8 \cdot 10^{-4}$	88	40	31

The incubation mixture contained ATP and adrenochrome in the concentrations mentioned. 0.5 ml 10% homogenate was added to a total volume of 3.0 ml.
Incubation for 20 min at 30° C.

DISCUSSION

While it is known that noradrenaline produces hyperglycemia by exerting a glycogenolytic effect on the liver, it appears more uncertain whether noradrenaline has any effect on the muscle metabolism. No changes in the content of muscle glycogen after injection of noradrenaline in rats could be found (SAHYUN AND WEBSTER[20], BLOOM AND RUSSELL[21]). The present experiments have clearly shown that noradrenaline exercises the same metabolic effects in the diaphragm as shown in the case of adrenaline (WALAAS AND WALAAS[1], WALAAS[17]), the effect of noradrenaline being, however, considerably weaker.

The relation between the hyperglycemic effect of adrenaline and noradrenaline *in vivo* after injection in whole animals (MCCHESNEY, MCAULIFF AND BLUMBERG[22]) corresponds to the relationship between the glycogenolytic effect of these catechol amines in isolated liver slices (SUTHERLAND AND CORI[23]). Approximately the same relationship between the effect of adrenaline and noradrenaline is demonstrated concerning the inhibition of glycogen synthesis and glucose uptake in the isolated

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diaphragm. The somewhat uncertain effect of noradrenaline on the muscle metabolism *in vivo* may therefore depend upon the fact that noradrenaline gives vasoconstriction in skeletal muscle (DUNCANSON, STEWART AND EDHOLM[24]). On the other hand, adrenaline gives a pronounced vasodilation in muscles (ALLEN, BARCROFT AND EDHOLM[25]).

The problem whether the metabolic effects of adrenaline are due to unchanged adrenaline or oxidation products of the latter, has been a subject of attention since the investigations by CORI, FISHER AND CORI [6]. The observation that adrenaline protected with anti-oxidants exerted a strong hyperglycemic response by injection, indicated that adrenaline was the active substance.

It is well known that adrenaline is easily oxidized in neutral solution. The first product of oxidation, adrenoerythrine, is a very unstable substance (BU'LOCK AND HARLEY-MASON[27]) with an absorption maximum at 250 mμ (RUIZ-GIJON[28]). It is easily converted further to the red adrenochrome with absorption maxima at 300 mμ and 485 mμ (LUND[11]). The possibility that adrenaline exercises metabolic effects only after oxidation to adrenochrome has received some attention since RANDALL[8] demonstrated that adrenochrome inhibited the glycolysis in brain homogenate due to inhibition of the hexokinase and phosphofructokinase reactions (MEYERHOF AND RANDALL[9]).

Our experiments have added evidence that the metabolic effects by adrenaline are due to the adrenaline itself. Thus, adrenaline protected with glutathione is metabolically active on the isolated diaphragm. The diaphragm *in vitro* prevents adrenaline from being oxidized probably because "protecting" substances are being liberated from the muscle. Homogenates or extracts made from the diaphragm also protect adrenaline from being oxidized. The spectrophotometric measurements performed indicate the formation of small amounts of adrenoerythrine when either glutathione or isolated diaphragm were added to the incubation medium containing adrenaline. As shown by MURACCIOLE AND RUIZ-GIJON[7] this compound has the same hyperglycemic effect as adrenaline on injection in rabbits. Possibly an equilibrium in the cell is set up between adrenaline and adrenoerythrine.

The inhibition of hexokinase activity in diaphragm extracts by adrenochrome may be due to reversible oxidation of sulfhydryl groups. The sensivity of hexokinase to SH-agents and the partial reversal of the inhibition exerted by adrenochrome on hexokinase activity by glutathione supports this assumption. The possible physiological importance of adrenochrome, however, on regulation of carbohydrate metabolism in muscle is not known. Adrenochrome is without hyperglycemic effect by injection *in vivo* (MURACCIOLE AND RUIZ-GIJON[7]) and has no effect *in vitro* on the isolated diaphragm. The question of penetration must be taken into consideration and the intracellular oxidation of adrenaline to adrenochrome must be considered a possibility (BACQ[29]). According to GREEN AND RICHTER[30] adrenaline is converted to adrenochrome by the cytochrome system. In unpublished experiments it has been shown by the authors that adrenaline is oxidized to adrenochrome by sarcosomes isolated from the rat diaphragm.

In conclusion it should be pointed out the essential results obtained. Adrenaline decreases glucose uptake in the isolated diaphragm but has only a slight inhibitory effect on hexokinase activity in extracts of the diaphragm. The reverse situation is shown when experiments with adrenochrome are performed. Adrenochrome inhibits

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hexokinase activity in extracts but has no effect on glucose uptake in the diaphragm.

More work is therefore required to establish the role of adrenochrome in regulation of carbohydrate metabolism in the intact muscle. So far, the experiments indicate that the inhibition of glucose uptake in the diaphragm by adrenaline is due to the unoxidized adrenaline molecule.

ACKNOWLEDGEMENT

We wish to thank Professor U. S. VON EULER for samples of noradrenaline and Eli Lilly and Company for a grant which made this work possible.

SUMMARY

1. Noradrenaline *in vitro* produced an inhibition of glycogen synthesis and glucose uptake in the diaphragm, *i.e.* the same effects at those produced by adrenaline. The relative biological activity of *l*-adrenaline, *l*-noradrenaline and *d,l*-noradrenaline was 1:1/4:1/10. This is a situation corresponding to that known for the glycogenolytic effect of these substances in the liver.

2. Adrenaline "protected" against oxidation by glutathione gave the same metabolic effects in the diaphragm as "unprotected" adrenaline. Adrenochrome has no metabolic effects on the diaphragm *in vitro*.

3. In saline phosphate medium, pH 7.4 at 37° C, adrenaline was rapidly oxidized to adrenochrome. This conversion was inhibited by glutathione or by addition of diaphragm to the incubation medium.

4. Hexokinase activity in extracts of rat diaphragm was sensitive to sulfhydryl agents Adrenochrome inhibited hexokinase activity probably by reversible oxidation of sulfhydryl groups.

5. The experiments indicate that the physiological effects of adrenaline on carbohydrate metabolism in muscle is due to the adrenaline molecule as such.

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