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Nature of the Stimulation of Biogenesis of Cholesterol in the Liver by Noradrenaline

By RAJAN GEORGE and T. RAMASARMA
Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

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1. Administration of noradrenaline increased the incorporation of [1-^{14}C]acetate into hepatic sterols and the activity of liver microsomal 3-hydroxy-3-methylglutaryl-CoA reductase. 2. The stimulation was observed at short time-intervals with a maximum at 4h and was progressive with increasing concentrations of noradrenaline. 3. Protein synthesis *de novo* was a necessary factor for the effect. 4. The stimulatory effect was not mediated through the adrenergic receptors, but appears to involve a direct action of the hormone within the hepatocyte.

Several hormones are known to influence the biogenesis of sterols in the liver and the activity of 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34), the rate-determining step in the pathway. An increase was observed after injection of pharmacological doses of insulin (Nepokroeff *et al.*, 1974), glucagon (Huber *et al.*, 1973) or tri-iodothyronine (Ness *et al.*, 1973). In experiments with rat liver slices, Bortz (1968) found that injection of noradrenaline resulted in a twofold stimulation of cholesterol biogenesis from [1-^{14}C]acetate, with a lag period of 12h. Noradrenaline did not increase the depressed activity of hydroxymethylglutaryl-CoA reductase in adrenalectomized rats (Edwards, 1973). Adrenaline and noradrenaline have been shown to increase the rate of incorporation of [1-^{14}C]acetate into non-saponifiable lipids and the activity of hydroxymethylglutaryl-CoA reductase in rat hepatocytes (Edwards, 1975).

A study of the effect of noradrenaline on hepatic cholesterol biogenesis and the activity of hydroxymethylglutaryl-CoA reductase in rat liver is reported here. The effect of different adrenergic-blocking agents on the stimulation of noradrenaline has been studied with a view to understanding the nature of this stimulatory effect. The results indicate that the effect of noradrenaline is a result of direct action of the molecule within the cell and may not involve the mediation of either α- or β-adrenergic receptors, nor of cyclic AMP.

Materials and Methods

Chemicals

[1-^{14}C]Acetate (specific radioactivity, 49mCi/mmol) was obtained from Bhabha Atomic Research Centre, Bombay, India. 3-Hydroxy-3-methyl[3-^{14}C]-glutarate (specific radioactivity, 7.7mCi/mmol) was purchased from New England Nuclear Corp., Boston, MA, U.S.A. After suitable dilution with non-radioactive compound, the glutarate was converted into its anhydride, and then into the CoA derivative as described by Louw *et al.* (1969). The specific radioactivity of 3-hydroxy-3-methylglutaryl-CoA used was 395d.p.m./nmol. [2-^{14}C]Mevalonic acid was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Cycloheximide was purchased from Upjohn Co., Kalamazoo, MI, U.S.A. The following compounds were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.: glucose 6-phosphate, glucose 6-phosphate dehydrogenase, $NADP^+$, dithiothreitol, bovine serum albumin, DL-*threo*-dihydroxyphenylserine, actinomycin D and non-radioactive mevalonic acid lactone. The other compounds used were obtained as follows: noradrenaline (Koch–Light Laboratories, Colnbrook, Bucks, U.K.), propranolol [1-isopropylamino-3-(2-naphthyloxy)propan-2-ol] (gift from Dr. R. M. Marchbanks, Institute of Psychiatry, London SE5 8AF, U.K.), practalol [4-(2-hydroxy-3-isopropylaminopropoxy)acetanilide] (gift from ICI, Alderley Park, Macclesfield, Cheshire, U.K.), phenoxybenzamine hydrochloride [*N*-(2-chloroethyl)-*N*-(1-methyl-2-phenoxyethyl)benzylamine hydrochloride] (gift from Smith, Kline and French, Bangalore, India). Pargyline hydrochloride [*N*-benzyl-*N*-methylprop-2-ynylamine hydrochloride] was obtained without charge from Abbott Co., North Chicago, IL, U.S.A., and compound Ro 4-1284 (2 - ethyl - 2 - hydroxy - 3 - isobutyl - 9,10 - dimethoxy - 1,2,3,4,6,7 - hexahydrobenzoquinolizene) was from Roche Products, Bombay, India.

Animals and treatment

Male albino rats obtained from the Institute colony weighing 150–180g were used. The animals were fed

ad libitum on a pellet diet obtained from Hindustan Lever, Bombay, India. All compounds were administered intraperitoneally in 0.9% NaCl, either in solution or in suspension and the control animals received equivalent amounts of 0.9% NaCl. Except for time studies, all other experiments were done at 2h time-intervals. Inhibitors of protein biosynthesis and adrenergic-blocking agents were injected 10min before noradrenaline. All killings were done between 10:00 and 11:00h to avoid interference of rhythmic variations. Four animals or more were used in each group and the mean ± S.E.M. values obtained by independent analyses were used for the calculation of P values by Student's t test.

Incorporation of [1-^{14}C]acetate and [2-^{14}C]mevalonate into hepatic sterols

The radioactive compounds were injected intraperitoneally, 30min before the animals were killed by cervical dislocation. The procedure for isolation of non-saponifiable lipids and their fractionation on 5% deactivated alumina was as described by Subba Rao *et al.* (1976)

Assay of hydroxymethylglutaryl-CoA reductase

Rats were killed by cervical dislocation, and the livers were excised and placed in an ice-cold solution containing 30mM-EDTA, 70mM-NaCl, 10mM-dithiothreitol and 50mM-potassium phosphate buffer (pH 7.4), minced and homogenized (5ml/g of liver) in a Potter–Elvehjem-type glass homogenizer. Microsomal fractions were prepared by the method of Shapiro & Rodwell (1971). The microsomal pellet was resuspended in the same homogenization medium and the suspension was used as the enzyme source. Assay of the enzyme was carried out by the micro-method of Shapiro *et al.* (1974). The enzyme (about 0.3mg of microsomal protein) was pre-incubated for 5min at 37°C with 4.5μmol of glucose 6-phosphate, 450nmol of $NADP^+$ and 1 unit of glucose 6-phosphate dehydrogenase in a total volume of 100μl. The reaction was started by adding hydroxymethyl[3-^{14}C]glutaryl-CoA and stopped by the addition of 30μl of 10M-HCl. Non-radioactive mevalonic acid (0.5mg) was added and the mixture was incubated for a further 30min at 37°C for complete lactonization of the mevalonic acid. The mevalonolactone was separated on silica-gel t.l.c. plates and the radioactivity determined as described previously (Subba Rao *et al.*, 1976). Protein was measured by the method of Gornall *et al.* (1949), with bovine serum albumin as standard. Specific activity of hydroxymethylglutaryl-CoA reductase is expressed as pmol of mevalonate formed/min per mg of microsomal protein.

Results

Effect of the administration of noradrenaline on biogenesis of sterols

After administration of noradrenaline (0.2mg/rat), the incorporation of [1-^{14}C]acetate into sterols showed a twofold increase in 2h, but the compound was without effect on the incorporation of [2-^{14}C]-mevalonate (Table 1). As there was no change in the incorporation rate of [2-^{14}C]mevalonate, further experiments were carried out with [1-^{14}C]acetate. These results suggest that the effect is at a pre-mevalonate site, and probably on the enzyme hydroxymethylglutaryl-CoA reductase. On testing the enzyme activity in noradrenaline-treated animals, the expected increase in the activity of hydroxymethylglutaryl-CoA reductase was found (Table 1).

Time-course of stimulation by noradrenaline

The incorporation of [1-^{14}C]acetate into hepatic sterols was determined at time-intervals after the

Table 1. *Effect of noradrenaline on the incorporation of [1-^{14}C]acetate and [2-^{14}C]mevalonate into sterols and on the activity of hydroxymethylglutaryl-CoA reductase*

Noradrenaline (0.2mg/rat) was given intraperitoneally to rats, as a solution in 0.9% NaCl, 2h before they were killed. Controls received injections of 0.9% NaCl. [1-^{14}C]Acetate (10μCi/rat) and [2-^{14}C]mevalonate (0.2μCi/rat) were given as intraperitoneal injections 30min before killing. Extraction and fractionation of sterols and the measurement of activity of hydroxymethylglutaryl-CoA reductase were carried out as described in the Materials and Methods section. Numbers in parentheses indicate the numbers of animals used. N.S., Not significant.

	Incorporation into sterols (d.p.m./g of liver)			
	−Noradrenaline	+Noradrenaline	Change (%)	P value
[1-^{14}C]Acetate	2718 ± 373 (4)	6221 ± 414 (4)	229	<0.01
[2-^{14}C]Mevalonate	3998 ± 216 (6)	4126 ± 469 (6)	103	N.S.
Hydroxymethylglutaryl-CoA reductase activity (pmol/min per mg of protein)	232 ± 26 (4)	401 ± 35 (4)	173	<0.01

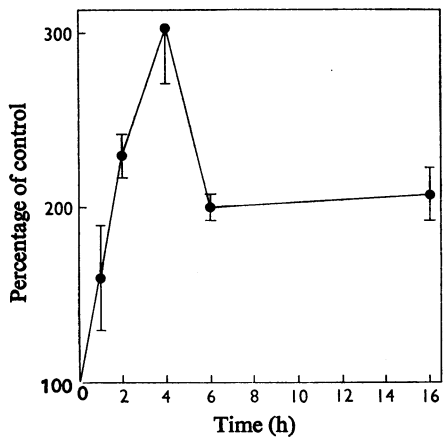

Fig. 1. *Time-course of stimulation of incorporation of [1-^{14}C]acetate into hepatic sterols in rats treated with noradrenaline*

The experimental group of rats received 0.2mg of noradrenaline as a solution in 0.9% NaCl, at the specified time-intervals. The control group received 0.9% NaCl alone. [1-^{14}C]Acetate (10μCi/rat) was injected intraperitoneally 30min before the rats were killed. At each point the values are compared with the respective controls and expressed as percentage of controls. Each point represents means ± S.D. obtained by independent analysis of livers from four animals in each group.

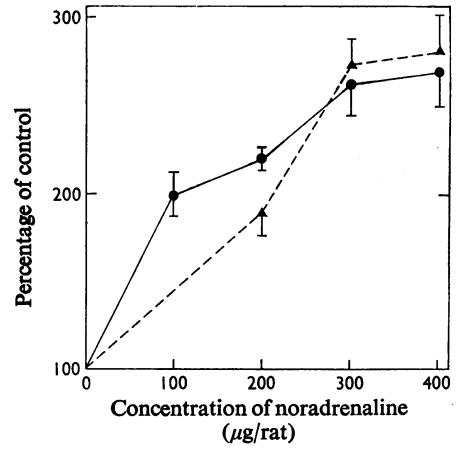

Fig. 2. *Effect of different concentrations of noradrenaline on the incorporation of [1-^{14}C]acetate into hepatic sterols and activity of hydroxymethylglutaryl-CoA reductase*

Groups of rats received intraperitoneal injections of the specified concentrations of noradrenaline as a solution in 0.9% NaCl, 2h before the animals were killed. The control group received 0.9% NaCl alone. All were given [1-^{14}C]acetate (10μCi/rat) intraperitoneally, 30min before being killed. Radioactivity incorporated into hepatic sterols and activity of hydroxymethylglutaryl-CoA reductase are given as percentage of controls. Means ± S.D. obtained by independent analysis of livers from four animals in each group are given. ●, Radioactivity incorporated into sterols (d.p.m./g of liver); ▲, specific activity of hydroxymethylglutaryl-CoA reductase (pmol/min per mg of protein).

administration of noradrenaline (0.2mg/rat). In each experiment, the result was compared with that of the control group, which received only 0.9% NaCl. The stimulatory effect could be observed even 1h after administration of noradrenaline, in contrast with the observation by Bortz (1968), where the effect was seen only after a lag period of 12h. Bortz (1968) used a liver-slice system, whereas whole animals were used in the present studies. Maximum incorporation was obtained by 4h. Thereafter it decreased and stabilized at a value higher than that of the normal (Fig. 1). For convenience, the 2h time-interval was chosen for other experiments.

Effect of different concentrations of noradrenaline

Radioactivity incorporated into hepatic sterols from [1-^{14}C]acetate and the activity of hydroxymethylglutaryl-CoA reductase were measured 2h after the administration of different concentrations of noradrenaline. The stimulation was progressive with increasing concentrations of the hormone for both biogenesis of sterols and hydroxymethylglutaryl-CoA reductase activity (Fig. 2). A concentration of 0.2mg of noradrenaline/rat was used in further experiments, as sufficient stimulation could be observed at this dosage, which is within physiological limits.

Effect of inhibitors of protein synthesis

To test whether the observed responses involved protein synthesis *de novo*, the effect of two inhibitors, cycloheximide and actinomycin D, was studied. Cycloheximide by itself increased the incorporation of [1-^{14}C]acetate into sterols (Table 2), which precluded any meaningful conclusion from this experiment. Actinomycin D inhibited the stimulation by noradrenaline to a significant extent (Table 2), suggesting that synthesis of protein *de novo* is necessary for the stimulation. Higher concentrations of actinomycin D were not tried, as the compound by itself is known to induce the enzyme activity (De Matteis, 1968).

Effect of agents that increase endogenous noradrenaline

As these results clearly showed that exogenous noradrenaline stimulated biogenesis of cholesterol, it was decided to test the effect of agents known to

Table 2. *Effect of inhibitors of protein synthesis on incorporation of* [1-^{14}C]*acetate into hepatic sterols and hydroxymethylglutaryl-CoA reductase activity, in rats treated with noradrenaline*

Cycloheximide (0.2mg/rat) and actinomycin D (0.15mg/rat) were given as intraperitoneal injections, 10min before injection of noradrenaline (0.2mg/rat). All injections were given in 0.9% NaCl, and animals were killed after 2h. [1-^{14}C]Acetate (10μCi/rat) incorporation was for 30min. Determinations of radioactivity in the hepatic sterol fraction and activity of hydroxymethylglutaryl-CoA reductase were carried out as described in the Materials and Methods section. Values are means ± S.E.M. from four animals in each group. *P* values are given for the difference between values from control and treated rats.

	[1-^{14}C]Acetate incorporation (d.p.m./g of liver)		
	−Noradrenaline	+Noradrenaline	*P* value
Control	2981 ± 486	4885 ± 518	<0.05
Cycloheximide	5040 ± 971	8514 ± 1274	
Control	2587 ± 407	6370 ± 549	<0.001
Actinomycin D	4180 ± 700	2995 ± 295	
	Hydroxymethylglutaryl-CoA reductase activity (pmol/min per mg of protein)		
Control	232 ± 26	401 ± 35	<0.05
Actinomycin D	237 ± 25	325 ± 8	

increase the concentration of endogenous noradrenaline. The main pathway for degradation of catecholamines is through monoamine oxidase. Pargyline (Hellerman & Erwin, 1968), the well-known monoamine oxidase inhibitor which prevents the degradation of noradrenaline, and the drug Ro 4-1284, which increases the concentration of noradrenaline at the site by enhancing the rate of release (Pletscher *et al.*, 1962), were used for this set of experiments. Results in Table 3 show that both the compounds tested effectively stimulated hepatic sterol biogenesis from acetate. When pargyline and noradrenaline were given together, there was no further stimulation over that obtained with pargyline alone. It is therefore possible that the endogenous amine concentration would have reached saturation in the presence of pargyline.

Effect of adrenergic-blocking agents

Effects of catecholamines are known to be mediated through the two adrenergic membrane receptors (α- and β-receptors) described in various tissues, including the liver (Hornbrook, 1970). Catecholamines are known to combine with either or both of these receptors on the cell membrane, resulting in specific cellular responses. Several pharmacological agents specifically inhibit responses to catecholamines. To discover if any of the adrenergic receptors are involved in the stimulation of sterol biogenesis by noradrenaline, a set of experiments using specific blocking agents was designed. The adrenergic-blocking agents are known to decrease the rate of release of noradrenaline from the nerve granules and inhibit the ATP-dependent uptake of the hormone (von Euler & Lishajko, 1966). Phenoxybenzamine, an α-adrenergic-blocking agent, and propranolol and practalol, both β-adrenergic-blocking agents, were used. The blocking agents were given to the animals 10min before injection of noradrenaline into the experimental group. The incorporation of [1-^{14}C]acetate into sterols and the activity of hydroxymethylglutaryl-CoA reductase in the liver were determined. Table 4 shows that neither the α- nor the β-adrenergic-blocking agents could abolish the stimulatory effect of noradrenaline. These results suggest that the stimulation by noradrenaline of hepatic biogenesis of sterols is not mediated by either α- or β-adrenergic receptors.

Effect of dibutyryl cyclic AMP

The results obtained from studies *in vivo* and *in vitro* of sterol biogenesis with cyclic AMP are contradictory. Bricker & Levey (1972) reported that cyclic AMP inhibited sterol biogenesis *in vitro*. Our finding (Subba Rao *et al.*, 1976) indicated that metabolites derived from ATP, including cyclic AMP, stimulated biogenesis of sterols in starved rats *in vivo*. It seemed worth while therefore to check the effect of cyclic AMP in the normally fed animals, in comparison with that in starved animals used in our previous experiments, even though the above experiments with β-adrenergic-blocking agents clearly showed that cyclic AMP may not be involved in the action of noradrenaline. It is clear from Table 5 that

Table 3. *Effect of agents that increase endogenous noradrenaline on the incorporation of* [1-^{14}C]*acetate into hepatic sterols*
All compounds were given intraperitoneally as solution in 0.9% NaCl. Controls received 0.9% NaCl. Pargyline (5mg/rat) was given 10min before injection of noradrenaline (0.2mg/rat). Animals were killed after 2h. [1-^{14}C]Acetate (10μCi/rat) was given as an intraperitoneal injection 30min before killing. Ro 4-1284 (2mg/rat) was given 2h before killing. Values are means ± S.E.M. Numbers in parentheses indicate the numbers of animals used.

Compound administered	Incorporation of [1-^{14}C]acetate into hepatic sterols (d.p.m./g of liver)	Change (%)	*P* value
0.9% NaCl	2516±661 (4)	100	—
Pargyline	7579±1523 (4)	301	<0.02
Noradrenaline	3964±386 (4)	158	<0.05
Noradrenaline+pargyline	7099±1190 (4)	282	<0.05
0.9% NaCl	2063±351 (5)	100	—
Ro 4-1284	5500±567	267	<0.001

Table 4. *Effect of adrenergic-blocking agents on the incorporation of* [1-^{14}C]*acetate into hepatic sterols and hydroxymethylglutaryl-CoA reductase activity in rats treated with noradrenaline*
Phenoxybenzamine (2mg/rat), practalol (5mg/rat) and propranolol (2.5mg/rat) were given intraperitoneally as a suspension in 0.9% NaCl 10min before injecting noradrenaline (0.2mg/rat). Animals were killed after 2h. [1-^{14}C]-Acetate (10μCi/rat) was given as an intraperitoneal injection 30min before killing. Radioactivity incorporated into hepatic sterols and the activity of hydroxymethylglutaryl-CoA reductase were determined as described in the text. The values are means ± S.E.M. for four or more animals in each group.

Experiment	Compound injected	−Noradrenaline	+Noradrenaline	Change (%)	*P* value
Incorporation of [1-^{14}C]acetate into hepatic sterols (d.p.m./g of liver)	0.9% NaCl	1894±211	6158±267	325	<0.01
	Phenoxybenzamine	2592±324	5702±888	220	<0.01
Hydroxymethylglutaryl-CoA reductase activity (pmol/min per mg of protein)	0.9% NaCl	232±26	401±35	173	<0.01
	Phenoxybenzamine	213±17	373±23	175	<0.01
Incorporation of [1-^{14}C]acetate into hepatic sterols (d.p.m./g of liver)	0.9% NaCl	1290±157	4941±903	383	<0.01
	Practalol	1577±252	4417±575	280	<0.01
Hydroxymethylglutaryl-CoA reductase activity (pmol/min per mg of protein)	Practalol	285±31	548±41	192	<0.01
Incorporation of [1-^{14}C]acetate into hepatic sterols (d.p.m./g of liver)	0.9% NaCl	2271±291	5095±435	224	<0.001
	Propranolol	2913±475	7168±1480	246	<0.01
Hydroxymethylglutaryl-CoA reductase activity (pmol/min per mg of protein)	Propranolol	267±16	584±69	218	<0.002

this compound was without effect on sterol biogenesis in fed animals *in vivo*. Theophylline, which is known to increase cyclic AMP by inhibiting phosphodiesterase (Sutherland *et al.*, 1968), also had no effect (Table 5). The possibility of cyclic AMP being a mediator in the stimulatory effect of noradrenaline seems remote, in view of the negative results in the experiments with the adrenergic-blocking agents, theophylline and exogenous cyclic AMP.

Effect of dihydroxyphenylserine

The above results suggested that the stimulatory action of noradrenaline could not be mediated through the adrenergic receptors or cyclic AMP, and

Table 5. *Effect of dibutyryl cyclic AMP and theophylline on incorporation of [1-^{14}C]acetate into hepatic sterols and activity of hydroxymethylglutaryl-CoA reductase*

Dibutyryl cyclic AMP and theophylline were given intraperitoneally, 2h before the rats were killed. [1-^{14}C]Acetate (10μCi/rat) was given 30min before the animals were killed. Experimental details are given in the Materials and Methods section. Values are means ± S.E.M. for four or more animals in each group. N.S., Not significant.

	Compound injected	−Noradrenaline	+Noradrenaline	Change (%)	*P* value
Incorporation of [1-^{14}C]acetate into hepatic sterols (d.p.m./g of liver)	0.9% NaCl	3910±656	—	100	—
	Cyclic AMP	5054±562	—	129	N.S.
Hydroxymethylglutaryl-CoA reductase activity (pmol/min per mg of protein)	0.9% NaCl	318±27	—	100	—
	Cyclic AMP	326±40	—	102	N.S.
Incorporation of [1-^{14}C]acetate into hepatic sterols (d.p.m./g of liver)	0.9% NaCl	2037±273	4752±391	233	<0.001
	Theophylline	1663±302	4266±908	258	<0.05

Table 6. *Effect of 3,4-dihydroxyphenylserine on the incorporation of [1-^{14}C]acetate into hepatic sterols and activity of hydroxymethylglutaryl-CoA reductase*

DL-*threo*-Dihydroxyphenylserine was given intraperitoneally to rats as a suspension in 0.9% NaCl, 2h before the animals were killed. [1-^{14}C]Acetate (10μCi/rat) was injected intraperitoneally 30min before the rats were killed. Radioactivity incorporated into hepatic sterols and hydroxymethylglutaryl-CoA reductase activity were determined as described in the Materials and Methods section. Values are means ± S.E.M. for four or more animals in each group. * P <0.01; ** P <0.05.

Treatment	Incorporation of [1-^{14}C]acetate into hepatic sterols (d.p.m./g of liver)	Hydroxymethylglutaryl-CoA reductase activity (pmol/min per mg of protein)
0.9% NaCl	2022±268	250±15
3,4-Dihydroxyphenylserine (5mg/rat)	3920±238*	387±57**
0.9% NaCl	1520±289	240±28
3,4-Dihydroxyphenylserine (10mg/rat)	4412±427*	462±21*

this opened the possibility that the action could be a direct one, at the intracellular level. To test this possibility, the effect of treatment with 3,4-dihydroxyphenylserine was tested. By the action of L-aromatic amino acid decarboxylase present in the cytosol (Dairman *et al.*, 1972) on this compound, the concentration of noradrenaline increased within the cells when administered to animals (McCann *et al.*, 1972). As expected, the compound stimulated both the incorporation of [1-^{14}C]acetate into hepatic sterols and the activity of hydroxymethylglutaryl-CoA reductase, at two concentrations tested (Table 6). These results further support the direct mode of action of noradrenaline molecules within the hepatocyte, unconnected with the adrenergic membrane receptors or the adenylate cyclase system, with respect to biogenesis of sterols.

Discussion

The foregoing experiments show that administration of noradrenaline stimulates hepatic biogenesis of sterols and hydroxymethylglutaryl-CoA reductase activity at shorter time-intervals than those observed by Bortz (1968). Maximum stimulation was observed after 12h in his experiments with liver slices, in contrast with 4h in the studies reported here.

On the basis of the present knowledge of the nature of action of noradrenaline, two alternatives could be considered for its stimulation of sterol biogenesis: either action at the cell-membrane level, involving specific adrenergic receptors (α or β), or a direct action at the intracellular level. The mechanism involving a membrane receptor is ruled out, as the adrenergic-blocking agents failed to prevent the stimulation of sterol biogenesis by the hormone. As the agents that increase endogenous noradrenaline, and more specifically 3,4-dihydroxyphenylserine, which gives rise to noradrenaline within the cell cytosol, elicited the same response as the hormone, the second alternative seems likely. A similar case has been reported for gluconeogenesis and glycogenolysis, where the action of the hormone persisted, even when the enhancement of cyclic AMP concentration was abolished by the adrenergic-blocking agents (Saitoh & Michio, 1976), which again shows that cyclic AMP may not be the mediator of this type of response to noradrenaline treatment.

Catecholamines are known to enhance lipolysis in intact rats, resulting in increased concentrations of free fatty acids in the liver. It was proposed by Bortz (1973) that the effects of catecholamines are mediated by free fatty acids. But from the experiments with rat hepatocytes, Edwards (1975) concluded that the above hypothesis may not be valid, as an increased supply of free fatty acids is impossible with isolated hepatocytes, and that the effect of catecholamines must be a direct one within the cells. Results of the present studies with adrenergic-blocking agents, cyclic AMP and 3,4-dihydroxyphenylserine, all favour the direct mode of action of noradrenaline within the hepatocyte.

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