EVIDENCE FOR SEPARATE EPINEPHRINE AND GLUCAGON RESPONSIVE ADENYL CYCLASE SYSTEMS IN RAT LIVER

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Adenyl cyclase plays a central role in the hormonal regulation of the activity of a number of intracellular enzymes. Certain questions are posed by the differences in structure of glucagon and epinephrine, the two hormones which regulate liver cyclase. This report presents evidence for two separate adenyl cyclase systems. This evidence includes selective destruction of the epinephrine cyclase activity, additive effects with combinations of the two hormones, and separation of the two enzymic activities by centrifugation.The data could also be interpreted in terms of separate systems which mediate the interaction between each hormone and adenyl cyclase.

Other investigators have favored a single cyclase hypothesis, since additive effects were not observed with saturating concentrations of the two hormones (Sutherland and Robison, 1966).It was also reported that in broken cell preparations the stimulation produced by glucagon was significantly greater than that produced by epinephrine (Makman and Sutherland, 1964).This in vitro observation of differences in hormonal activity contradicts the known efficacy of the two hormones in intact cell preparations (Levine, 1965).

METHODS: Male Sprague Dawley rats (100 to 150 grams) were anaesthetized with ether and exsanguinated. The livers were perfused in situ with cold buffer, excised, minced and disrupted with a teflon/glass Potter-Elvehjem homogenizer. C^{14}-ATP, used as substrate, was incorporated into a reaction volume of 20 microliters. Incubations were terminated with 20 microliters of a mixture containing 15% TCA, and unlabelled and tritiated cyclic AMP. C^{14}-cyclic-AMP, synthesized by liver enzymes, was isolated from the mixture of labelled products by descending chromatographic separation on Ecteola thin layers. Development in the first solvent system (propanol/ammonia/water, 6:3:1) took 21 hours. The plates were then shaved proximal to the cyclic-AMP markers, rewicked and run for 18 hours in the second solvent system (glacial acetic acid/butanol/water, 1:2:1). C^{14}-labelled cyclic-AMP, isolated by this method, exhibited chromatographic, spectrophotometric and biochemical properties identical with those of crystalline cyclic-AMP.

RESULTS: The response of whole-homogenate cyclase to glucagon in vitro was consistently less than that of repeatedly washed membrane particles derived from the same homogenate (Table 1). The initial rates produced by washed particles were greater than those of the whole homogenate, due to the more rapid depletion of substrate and hormone by whole homogenate enzymes (Fig. 1). Glucagon responsive cyclase activity increased after washing, primarily because soluble phosphodiesterase was removed. However, those particles in which the response to glucagon had been enhanced by repeated washing had lost virtually all responsiveness to epinephrine (Table 1). This observation prompted an examination of the effects of serial dilution on the responsiveness of whole homogenate preparations to

TABLE I

Response of adenyl cyclase to glucagon and L-epinephrine.

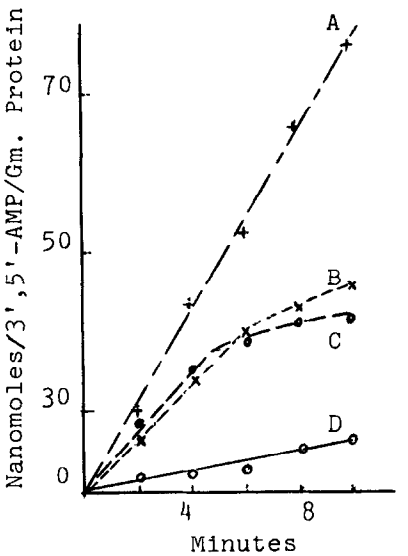
	HOMOGENATE*	WASHED PARTICLES*	WASHED PARTICLES PLUS SUPERNATANT*
NO HORMONE:	7.1 (5.5-8.6)	3.2 (1.8-3.8)	4.3 (3.5-5.2)
EPINEPHRINE:	20.8 (17.3-25.0)	4.2 (3.5-4.6)	4.8 (4.1-6.1)
GLUCAGON:	23.0 (18.3-24.5)	35.4 (32.0-45)	17.5 (14.5-19.3)

*Nanomoles of 3',5'-AMP synthesized/gm. protein/10 min. The averages of six experiments are given and the values in parentheses are the range limits in each group.

Reaction mixtures contained, in a volume of 20 microliters: 0.045M PO_4 buffer(pH 7.5), 3.5 x 10^{-3} M $MgSO_4$, 4 x 10^{-2}M glycylglycine, 10^{-4}M EDTA, 6.6 x 10^{-3}M Aminophylline, 2 x 10^{-2}M PEP, 2 micrograms of PEP-Kinase(Sigma Type II), and 5.5 x 10^{-4}M C^{14}-ATP. Concentration of whole liver homogenate was adjusted to 800 micrograms per 20 microliters. Protein concentrations of washed particle suspensions were adjusted to the equivalent of 800 micrograms/20 microliters (in the whole homogenate enzyme). Hormone concentrations were 1.8 x 10^{-6} M glucagon or 8 x 10^{-6}M L-epinephrine bitartrate. Mixtures were incubated for 6 min. at 30°.

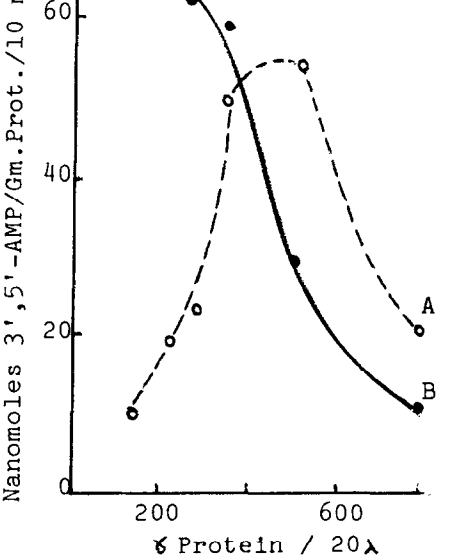
epinephrine and glucagon, since consequences of serial dilution are similar to those of repeated washing. With dilution of the whole homogenate enzyme from 800 to 600 micrograms/20 microliters, the epinephrine cyclase exhibited the same apparent stimulation of specific activity as did the glucagon responsive cyclase. Beyond this point, however, dilution resulted in rapid loss of epinephrine cyclase activity(Fig. 2). Once epinephrine cyclase activity had been lost as a result of either washing or dilution, resuspension of the particles in high speed supernatants from the whole homogenate (in effect a reversal of the dilution and washing procedures) did not restore the epinephrine responsive adenyl cyclase activity (Table 1).

FIGURE 1



Response of cyclase to glucagon and L-epinephrine with whole homogenate and washed particle enzymes. Particles were washed x 3 by suspension and sedimentation (8700 x g for 5 min.) at 4°. Conditions and reactant concentrations were as in Table 1. A=washed particles and glucagon. B=homogenate with epinephrine. C=homogenate with glucagon. D=washed particles with epinephrine.

FIGURE 2



Response of liver adenyl cyclase to glucagon and L-epinephrine with varying homogenate concentrations. Assay conditions and reactant concentrations were as shown in Table 1.
A= epinephrine.
B=glucagon.

Relative to glucagon responsive cyclase, epinephrine responsive cyclase was found to be more easily damaged by heating or sonication. Following incubation at 37° for 18 minutes the response to epinephrine had fallen more than 80%, whereas the response to glucagon was undiminished. Following sonication (100 watts for 20 seconds) the response to epinephrine had fallen more than 50%, whereas the response to

TABLE II

Additive effects with epinephrine and glucagon in combination.

Experiment #	ΔGE†	Δ(G + E)@	[GE/(G + E)] x 100
1.	23.3*	28.8*	83%
2.	25.2	22.2	113%
3.	18.4	24.8	74%
4.	33.0	29.4	112%
5.	29.4	24.5	120%
6.	30.1	26.2	87%
7.	31.1	32.4	96%

*Nanomoles of 3',5'-AMP synthesized/gm.protein/10 min.
† ΔGE=increase in adenyl cyclase activity stimulated by epinephrine and glucagon in combination.
@ΔG+ E=sum of adenyl cyclase activity stimulated by glucagon and epinephrine used separately.

L-epinephrine bitartrate (8 x 10^{-6} M) and glucagon (1.8 x 10^{-6} M) were present as indicated. Reaction components and conditions were as described in Table I.

TABLE III

The centrifugal behavior of epinephrine and glucagon cyclase.

g Forces	PERCENT ACTIVITY REMAINING SUSPENDED: Epinephrine		Glucagon	
	Av.	Range	Av.	Range
0	100		100	
121	92	(85-100)	90	(81-99)
480	73	(51-97)	15	(11-17)
755	47	(35-52)	4	(0-9)

The ice cold phosphate homogenates were filtered through #40 stainless steel mesh and centrifuged at the indicated g forces in a Sorvall at 4° for 5 min. Aliquots of supernatants were removed following each centrifugal step and enzyme activity determined under the assay conditions described in Table I. Averages and ranges for six experiments are given.

glucagon had fallen only 15%.

When both hormones were added in combination at saturating concentrations, the rates closely approximated the sum of the rates associated with stimulation by the individual hormones (Table 2).

Though the glucagon and epinephrine cyclase activities

sedimented at moderate g forces, there were differences in their rates of sedimentation. In homogenates prepared in phosphate buffer, epinephrine responsive cyclase was consistently associated with a more slowly sedimenting population of membrane particles (Table 3).

DISCUSSION: It would seem consistent with the above data to propose the existence of two completely separate adenyl cyclase systems in liver which respond independently to specific hormones. The epinephrine sensitive cyclase undergoes irreversible denaturation upon dilution of soluble factor(s) in the homogenate. Although soluble factor(s) would appear necessary to maintain the integrity of the epinephrine cyclase, there is at present no evidence to suggest that such factor(s) are in any way necessary for the effective interaction of epinephrine with cyclase.

Previous examination of the question of heterogeneity of cyclase revealed that hormones in combination did not produce additive effects (Sutherland and Robison,1966). Unless precautions were taken to prevent dilution of factor(s) necessary for the preservation of epinephrine cyclase in experiments using broken cell preparations, damage of this enzyme could occur during the course of experimental manipulation. Earlier reports of large differences between glucagon and epinephrine sensitive adenyl cyclase activities could have resulted from such inadvertent trauma to the epinephrine cyclase (Makman and Sutherland, 1964).

It is unlikely that the two cyclase systems are segregated in different types of cells, since both glucagon and epinephrine are capable of completely depleting glycogen stores in perfused liver (Levine,1965). It is improbable that cyclic-

AMP can arise in one cell and function in an adjacent cell in view of the relative impermeability of membranes to cyclic-AMP (Posternak,Sutherland and Henion,1962). While response to glucagon is essentially limited to liver, epinephrine elicits responses in many other tissues as well. The advantages of a double cyclase system in liver would appear to derive from the greater flexibility of response which it allows.It cannot as yet be assumed that the hormones act directly upon adenyl cyclase. Our results may also be interpreted in terms of separate (membrane-associated) systems which mediate the interaction between each hormone and the enzyme.

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References:

Levine,R.A.,Am.J.Physiol.,208:317 (1965).
Makman,M.H. and Sutherland,E.W.,Endocrinology,75:127 (1964).
Posternak,T.,Sutherland,E.W.,and Henion,W.F.,Biochem.Biophys. Acta, 65:558 (1962).
Sutherland,E.W. and Robison, A.G.,Pharmacol.Rev.,18:145(1966).