ESTERS OF ADENOSINE 5'-PHOSPHATE AS COPRODUCTS OF ADENOSINE 3',5'-CYCLIC PHOSPHATE IN REACTIONS OF ATP WITH ADENYLATE CYCLASE IN THE PRESENCE OF HYDROXY DERIVATIVES

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In the presence of high concentrations of some hydroxy derivatives, adenylate cyclase preparations were found to synthesize in addition to adenosine 3',5'-cyclic phosphate (cyclic AMP) also nucleotides identified as esters of adenosine 5'-phosphate. The formation of the latter esters predominates in the case of the solubilised or immobilised enzyme. An idea is discussed that the two reactions, both the formation of the cyclic and the formation of the acyclic phosphodiester, are catalysed with the same enzyme.

In the adenylate cyclase activity assays, the following techniques have been widely used: isolation of the radioactive product *via* Dowex 50 (H^+) ion exchange resin and negative precipitation on $BaSO_4$–$Zn(OH)_2$ (ref.[1]) or chromatography on a thin layer of cellulose[2]. In some communications on the use of the former method[3–6], the addition of various alcohols to incubation mixtures has been claimed to stimulate the formation of the cyclic AMP. Since the amount of the radioactive product formed by various adenylate cyclase preparations in the presence of hydroxy derivatives exceeded in some cases the amount of cyclic AMP after stimulation with sodium fluoride, it was questionable whether the radioactive product measured after the stimulation with hydroxy derivatives is a pure cyclic AMP. As demonstrated by Hynie and coworkers[7], the new nucleotide produced by adenylate cyclase preparations in the presence of sorbitol in addition to cyclic AMP, does not undergo degradation by the action of phosphodiesterase specific for 3',5'-cyclic phosphates. Recently, analogous findings on unknown nucleotides have been reported[8,9].

In the present paper, we wish to describe experimental conditions for the production of novel nucleotides by adenylate cyclase preparations in the presence of alcohols and separation of these nucleotides from cyclic AMP on alumina by the method of Ramachandran[10]. As suggested by comparison with authentic methyl and ethylene glycol ester of adenosine 5'-phosphate, it appears highly probable that the novel

nucleotides produced by adenylate cyclase preparation in the presence of hydroxy derivatives, are esters of adenosine 5'-phosphate.

EXPERIMENTAL

Adenosine 5'-[α-^{32}P]triphosphate sodium salt (0·5—3·0 mCi/mmol), [2-^{3}H]adenosine 5'-triphosphate ammonium salt (3·2 Ci/mmol) and [8-^{3}H]adenosine 3',5'-cyclic phosphate ammonium salt (12 Ci/mmol) were products of The Radiochemical Centre, Amersham, England. Adenosine 5'-triphosphate (trisodium salt), 2-phosphoenol pyruvate (tricyclohexylammonium salt), rabbit muscle pyruvate kinase (300 I.U. per ml, per 9·3 mg of the protein), and rabbit muscle myokinase (660 I.U. per 1 mg of the protein) were products of Calbiochem, Los Angeles, U.S.A. Triton X-100 and Lubrol PX (polyoxyethylene 23 lauryl ether) were products of Sigma, St. Louis, U.S.A. The cyclic AMP was purchased from Lachema, Brno, Czechoslovakia. Sepharose 4B and Sephadex G 100 were products of Pharmacia Fine Chemicals, Uppsala, Sweden. Neutral alumina for chromatographic adsorption analysis (Brockmann activity II) was purchased from Reanal, Hungary. Prostaglandin E_1 (PGE_1) was a gift of Dr J. E. Pike, Upjohn Company, Kalamazoo, U.S.A.

Adenosine 5'-Phosphate [P-Methyl Ester]

A solution of 2',3'-di-O-acetyl-N^6-acetyladenosine 5'-phosphate pyridinium salt (0·1 mmol) in pyridine (10 ml) is evaporated and to the residue there is added 1M solution of methanol in pyridine (0·2 ml), pyridine (1 ml), and 2,3,5-triisopropylbenzenesulfonyl chloride (60 mg). After 20 h, conc. aqueous ammonia is added. After additional 20 h, the mixture is chromatographed on 2 sheets of paper Whatman 3 MM in the solvent system 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2). The UV-absorbing bands (R_F 0·50) are eluted with 1% aqueous ammonia. The eluate is freeze-dried to afford the ammonium salt of the title ester (34 mg).

Adenosine 5'-Phosphate [P-Ethylene Glycol Ester]

The title compound was prepared analogously to the methyl ester starting from O-monomethoxytrityl ethylene glycol[11]. After the deblocking with ammonia, the mixture was evaporated and the residue deblocked by the action of 80% aqueous acetic acid (at 20°C, 4 h). The preparative paper chromatography was performed analogously to the preceding example to afford 25 mg of the title ester (R_F 0·42).

Adenylate Cyclase Preparations

The activity of adenylate cyclase was determined in the tissue homogenate prepared by a mild homogenisation of rat tissues in an all-glass homogeniser with the use of 75 mM-Tris-HCl buffer solution (pH 7·5) in the presence of 12·5—17·5 mM Mg^{++} and, in some cases, 1 mM EDTA. The homogenisation was performed at 0—4°C and the subsequent handling of the enzyme preparation was carried out at the same temperature. Plasmatic membranes were obtained in some experiments by repeated washings of the tissue homogenate with the above homogenisation mixture and centrifugation at 1000 *g*. These preparations were solubilised by the addition of Lubrol PX or Triton X-100 and then centrifuged at 12000 *g*. In some cases, the samples were filtered through Millipor or Synpor 0·2—0·4 μm filters or purified on Sephadex G-100. In some experiments, the solubilised material was bound to Sepharose 4B. The protein content of enzyme preparations was determined (Lowry[12]) and adjusted to the value of 40—80 μg per 20 μl.

Preparation of the Immobilised Enzyme

Activation of Sepharose 4B with cyanogen bromide and preparation of spacer for binding of solubilised enzymes were performed according to Cuatrecasas[13] with small modifications. For a direct binding of the solubilised enzymes there was used 100 mg of cyanogen bromide for activation of 1 ml of Sepharose 4B. To 3—5 ml of activated Sepharose, washed with physiological solution with phosphate buffer (pH 7·5) there was added 1 ml of the solubilisate and the mixture kept at 4°C overnight under occasional stirring.

Binding of solubilised enzymes with the use of a spacer was performed as follows. For activation of 1 ml of Sepharose 4B there was used 250 mg of cyanogen bromide. Hexamethylene--Sepharose 4B was prepared by adding an aqueous solution of 2 mmol of hexamethylenediamine (previously adjusted to pH 10 with hydrochloric acid) to 1 ml of activated Sepharose. By the action of O-bromoacetyl-N-hydroxysuccinimide on the washed hexamethylenediamine-Sepharose 4B, the bromoacetylaminohexyl-Sepharose 4B was prepared. After washing with a hydrogen carbonate solution of pH 9, 3—5 ml of the thus-prepared bromoacetylaminohexyl-Sepharose 4B were treated with 1 ml of the solubilisate, the whole mixture kept in an ice-box for 2 days, washed with a homogenisation mixture for adenylate cyclase, and stored at 4°C.

Adenylate Cyclase Activity Assays

The activity of adenylate cyclase was determined according to Krishna and coworkers[1] using modifications of Hynie and Sharp[14]. To 30 μl of the incubation mixture containing 1 μCi of ATP--[α-^{32}P] or ATP-[2-^{3}H] there was added 20 μl of the enzyme preparation or 50 μl of the preparation bound to Sepharose (corresponding to 20 μl of the enzyme protein) and the mixture was incubated at 37°C for 10—20 min. Unless stated otherwise, the final composition of the incubation mixture was as follows: ATP, 0·1 mM; Tris-HCl buffer solution, 30 mM (pH 7·5); cyclic AMP, 0·1 mM; Mg^{2+}, 5·0—7·5 mM; K^{+}, 5 mM; EDTA, 0·4 mM; phosphoenol pyruvate 5 mM; phosphoenol pyruvate kinase, 40 μg/ml; myokinase, 20 μg/ml. In each experiment, blanks were determined in the absence of the enzyme preparation. The reaction was stopped by the addition of 1 ml of 0·05M-HCl containing 50 μg of the cyclic AMP or [^{3}H]cyclic AMP (3000—5000 c.p.m.) for calculations of the cyclic AMP recovery. When the reaction was finished, the samples were immediately immersed for 5 min into boiling water.

The content of test tubes was chromatographed by two methods: *1*) On a column (4 cm × × 0·6 cm) of Dowex 50 W-X2 (200—400 mesh; H^{+} form) ion exchange resin according to Krishna and coworkers[1]. The columns were eluted with water and the effluent fraction containing the cyclic AMP (4th—5th ml) was purified by two precipitations with $BaSO_4$—$Zn(OH)_2$ at pH 7. *2*) On alumina according to Ramachandran[10]. After the termination of the incubation of adenylate cyclase, 1 ml of the solution was applied to a column (4·0 × 0·6 cm) of alumina (1 g). The cyclic AMP was eluted with 2·5 ml of 0·1M-Tris-HCl buffer solution at pH 7·5 (ref.[15]). Comparable results were obtained by the two methods with recovery between 70—90%.

The rat brain phosphodiesterase was prepared according to Brooker and coworkers[16]. In an experiment in which decomposition of adenylate cyclase reaction products was studied, cyclic AMP was added at the end of the incubation to the final 0·1 mM concentration, and the samples were reincubated at pH 7·5 for 20 min. After this period of time, the samples were boiled again, treated with additional cyclic AMP to determine the recovery, and worked up by the standard procedure according to ref.[1].

Paper chromatography of nucleotides after evaporation of the samples was performed on paper Whatman No 3 by the descending technique for 20 h in the solvent mixture 60 : 3 : 30 : 30 isobutyric acid–conc. aqueous ammonia–water–ethanol.

TABLE I

Formation of Radioactive Products (pmol/mg of the protein per 20 min) by Reaction with Adenylate Cyclase Preparations from Rat Tissues

Homogenate[a]	Blank[a]	NaF, $1 . 10^{-2}$M	Sorbitol, 1M
Heart	143	860	286
Liver	116	357	2 025
Epididymal fat	77	183	920
Jejunum	43	514	4 130
Brain	290	2 670	272

[a] Rat fat tissues were homogenised in 75 mM Tris-HCl buffer solution with 12·5 mM $MgCl_2$; an aliquot of the crude homogenate containing 50—60 μg of the protein was applied to assays of adenylate cyclase with the use of [α-^{32}P]-ATP in concentration of 10^{-4}M; the radioactive product was isolated according to the method of Krishna and coworkers[1]. The data represent average values from two experiments.

TABLE II

Formation of Radioactive Products (pmol/mg of the protein per 15 min) by Reaction with Adenylate Cyclase Preparations from Rat Liver and Jejunum Epithelial Cells in the Presence of Some Alcohols

Additions[a]	Liver	Jejunum
Blank[a]	100	30
Glucagon, 10^{-5}M	625	—
PGE_1, 10^{-5}M	—	130
NaF, 10^{-2}M	750	350
Methanol, 20%	700	170
Ethanol, 20%	0	20
Glycerol, 20%	1 325	1 350
Sorbitol, 2M	350	1 100

[a] Rat liver and jejunum epithelial cells were homogenised in 75 mM Tris-HCl buffer solution with 17·5 mM Mg^{2+} and 0·4 mM-EDTA; an aliquot of the homogenate containing 50—100 μg of the protein was applied to activity assays of adenylate cyclase; the product was isolated according to the method of Krishna and coworkers[1]; average values from two experiments are given.

Radioactive products were measured on Model 3390 Packard-Tricarb scintillation spectrometer. Aqueous solutions were measured with the use of the scintillation liquid SLD-31 (Spolana, Neratovice, Czechoslovakia); the ^{32}P radioactivity was in some cases measured by the Cerenkov radiation[17]. The chromatographic paper radioactivity was measured in the toluene scintillation solution SLT-31 (Spolana, Neratovice, Czechoslovakia).

RESULTS AND DISCUSSION

In examinations of the adenylate cyclase activity according to the method of Krishna and coworkers[1] which comprises isolation of the product on Dowex 50 (H^+) ion exchange resin and the subsequent double precipitation by means of $BaSO_4$–$Zn(OH)_2$, the preliminary experiments have shown that an increased amount of the radioactive product is formed with some rat tissues in the presence of 1M sorbitol. In most cases, the amount of the product exceeded the amount of cyclic AMP after stimulation of the enzyme with sodium fluoride. The highest formation of the product held initially to be the cyclic AMP, has been observed in the liver and intestinal epithelium (Table I). Consequently, the action of some other alcohols was tested using these two tissues (Table II). It has been thus confirmed that all the applied alcohols (except for ethanol) were able to increase the formation of radioactive products (determined by the above mentioned method[1]). These findings complete the earlier observations made in this laboratory[7] and are in accordance with observations of other authors[8,9].

The intensive formation of a radioactive product by adenylate cyclase preparations in the presence of various alcohols and the observation that these products are adsorbed on alumina[10] (Table III) led us to the conclusion that coproducts are involved which are determined by the loc. cit. method[1] together with the cyclic AMP. Petrack and coworkers[8,9] arrived to the same conclusion with the use of an additional separation of the product by paper chromatography.

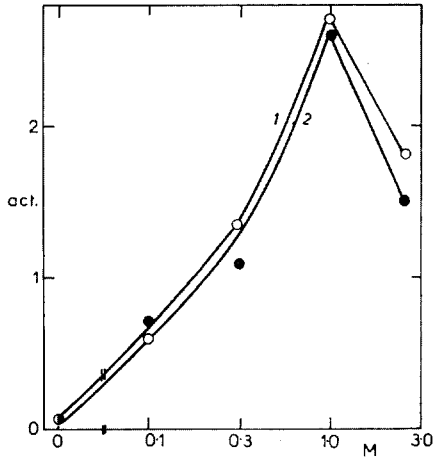

FIG. 1

Formation of Radioactive Products by Liver Adenylate Cyclase Preparations in the Presence of Increasing Concentrations of Sorbitol and with the Use of [α-^{32}P]-ATP (○) and [^3H]-ATP (●) as Substrate

For assay conditions see Table II. Average values are given from two experiments in nmol of the product per mg of the protein (20 min).

In the following part of our experiments, the character of substances produced by adenylate cyclase preparations in the presence of various hydroxy derivatives and optimum conditions for this production have been examined. The radioactive product resulting in the presence of sorbitol by the action of liver adenylate cyclase preparations, is formed both from [α-^{32}P]-ATP and from [^{3}H]-ATP labelled at position 2 of adenine (Fig. 1). It may be thus inferred that the nucleotide contains both adenine and a phosphate residue in position α. The same conclusion has also been drawn by Petrack and coworkers[9]. These authors have shown that the product does not contain incorporated beta or gamma phosphate and that ATP in the examined reaction may be partly replaced by CTP but not by UTP or GTP. On the other hand, according to our observations on guanylate cyclase activity (determined by our method[17]), analogous derivatives which do not separate from the cyclic GMP (ref.[18]) are formed even from GTP, in the presence of sorbitol.

The radioactive substance produced by adenylate cyclase preparations in the presence of sorbitol did not undergo degradation by the action of specific phosphodiesterase (Table IV).

TABLE III

Formation of a Radioactive Product (pmol/mg of the protein per 20 min) by Reaction with Adenylate Cyclase Preparations from Rat Liver and Jejunum Epithelial Cells in the Presence of Methanol and Ethylene Glycol and Separation of this Product from Cyclic AMP on Alumina Columns

Additions[a]	Isolation on Dowex 50 ion exchange resin + two $BaSO_4$–$Zn(OH)_2$ precipitations	Isolation on alumina
Liver homogenate		
Blank	79	70
NaF, 10^{-2} M	375	370
Methanol, 20%	757	20
Ethylene glycol, 20%	1 584	60
Jejunum homogenate		
Blank	61	40
NaF, 10^{-2} M	390	410
Methanol, 20%	373	35
Ethylene glycol, 20%	4 875	18

[a] The activity of adenylate cyclase in rat liver and jejunum epithelial cells was determined under conditions given in Table II: Average values from three experiments are given.

TABLE IV

Effects of Specific Phosphodiesterase on the Authentic [^{3}H]-Cyclic AMP and Product of the Liver Adenylate Cyclase Preparation in the Presence of Sorbitol

Substrate[a]	Phosphodiesterase	% of the substrate[b] (n)
[^{3}H]-cyclic AMP	−	100
	+	4·1 ± 1·0 (5)
[^{3}H]-product in the presence of 1M sorbitol	−	100
	+	95 ± 8·5 (6)

[a] The activity of adenylate cyclase was determined under conditions given in Table II. When the incubation was finished, the samples were boiled, and treated with cyclic AMP in the concentration 10^{-4}M. The control samples were then treated with [^{3}H]-cyclic AMP. The samples were incubated for additional 20 min in the presence of phosphodiesterase (pH 7·5), boiled again, and treated with additional non-radioactive cyclic AMP for purposes of recovery determination. The isolation was performed according to the standard method of Krishna and coworkers[1]. [b] Standard deviation is given: n designates the number of experiments.

TABLE V

R_F Values of Adenine Nucleotides and Products of Rat Tissue Adenylate Cyclase Obtained in the Presence of Various Alcohols and Isolated by Paper Chromatography

Nucleotide[a]	R_F	Nucleotide[a] (alcohol present)	R_F
ATP	0·13—0·15	(methanol)	0·55
ADP	0·26—0·27	(ethylene glycol)	0·49—0·50
AMP	0·41—0·43	(glycerol)	0·43
cyclic AMP	0·54—0·55	(sorbitol)	0·28—0·30

[a] The activity of adenylate cyclase was determined under conditions stated in Table II. The radioactive substances produced by adenylate cyclase preparations in the presence of various alcohols were isolated according to the method of Krishna and coworkers[1]. In an aliquot, the radioactivity was measured directly; another aliquot was evaporated, the residue applied to Whatman No 3 paper, and chromatographed by the descending technique for 20 h in the 60 : 3 : 30 : 30 isobutyric acid–conc. aqueous ammonia–water–ethanol mixture. Spots were identified under UV light and the radioactivity in the corresponding region was determined in the toluene scintillation solution.

Concerning the identification of substances produced by adenylate cyclase preparations in the presence of various hydroxy derivatives, it is not possible to prove directly incorporation of the alcohol residue into the nucleotide molecule by the addition of radioactive alcohols since the total radioactivity would exceed the technical scope of the determination. An indirect evidence on incorporation of the alcohol molecules into the newly formed nucleotides was inferred from chromatographic determinations. The radioactive substances produced by adenylate cyclase preparations in the presence of various alcohols were purified on Dowex 50 ion exchange resin and double precipitation with the use of $BaSO_4$–$Zn(OH)_2$ and then subjected to descending chromatography on paper Whatman No 3 for 20 h. Table V shows the R_F values of adenine nucleotides and derivatives produced in the presence of various alcohols. The mobility of these new nucleotides depends on the character of the alcohol incorporated. Except for the derivative obtained in the presence of methanol (the mobility of this derivative is similar to that of cyclic AMP), the R_F values of the other nucleotides are different.

The identification was performed by comparison of radioactive substances produced by reaction of adenylate cyclase preparations in the presence of methanol and ethylene glycol with an authentic methyl ester and ethylene glycol ester of adenosine 5′-phosphate. In the separation on Dowex 50 ion exchange resin and double precipitation on $BaSO_4$–$Zn(OH)_2$, the behaviour of these two esters is in principle identical with that of the cyclic AMP. On the other hand, the two alcohol esters were separated from the cyclic AMP by isolation on alumina. In the paper chromatography in the solvent system shown in Table V, the radioactivity of substances produced by reaction of adenylate cyclase preparations in the presence of methanol and ethylene glycol was localised in bands analogous to the UV-absorbing bands of authentic adenosine 5′-phosphate methyl ester and ethylene glycol ester. In the case of adenosine 5′-phosphate methyl ester, the cyclic AMP region exhibited about 95% of the total activity (determined by the method of Krishna and coworkers[1]) while in the case of 5′-AMP ethylene glycol ester, the cyclic AMP region showed only about 5 – 10% of the total radioactivity, the remaining 90 – 95% being concentrated in the R_F region of the 5′-AMP ethylene glycol ester. It may be thus inferred that the novel substances produced by reaction of adenylate cyclase preparations in the presence of methanol or ethylene glycol are the esters of adenosine 5′-phosphate. The nucleotides produced in the presence of other hydroxy derivatives may be consequently assumed to possess an analogous structure.

In conclusion, we wish to mention some earlier findings completing the present results. In adenylate cyclase preparations, the enzyme activity producing the cyclic AMP and the esters of adenosine 5′-phosphate is bound to the membrane fraction of cell homogenisates. The two enzymatic activities are equally dependent upon the concentration of the protein added and the optimum pH is between 7·5 and 8·0. Similarly to adenylate cyclase alone, the optimum production after the addition

of sorbitol was in the presence of 5 mM Mg^{2+} or Mn^{2+}. The nonspecific inhibitors of the hormonal and fluoride stimulation of adenylate cyclase, such as the pyrophosphate and Ca^{2+}, did not, however, affect the formation of the alcohol esters of 5'-AMP.

An addition of nonionic detergents (0·1 – 1·0% of Triton X-100 or Lubrol PX) to adenylate cyclase preparations results in solubilisation of the two enzymatical activities. It has been observed[7,18] that the two activities are present in the supernatant after the centrifugation at 12 000 *g*, and that they remain partly intact after filtration through a Millipor filter (0·2 – 0·4 μm) and after passage through a Sephadex G 100 column. By the addition of detergents, the two enzyme activities are affected in a different manner. Thus, an almost 100% antagonisation of the stimulative effect of hormones has been observed after the addition of detergents to homogenates of various rat tissues; the effect of sodium fluoride was considerably reduced and the basal activity of adenylate cyclase was affected depending on the type of the tissue and the type and concentration of the detergent (*cf.* Table VI and ref.[18]). On the other hand the enzymatic activity stimulated by various alcohols was in most cases increased by the addition of detergents (Table VI). The effect of sorbitol remained

TABLE VI

Effect of Lubrol PX on the Formation of Cyclic AMP and on the Formation of Products by Adenylate Cyclase Preparations in the Presence of Various Alcohols

Additions[a]	Isolation on Dowex + two $BaSO_4$-precipitations		Isolation on alumina	
	Lubrol PX absent	Lubrol PX present	Lubrol PX absent	Lubrol PX present
Blank	27 ± 3	0 ± 1	41 ± 2	0 ± 1
Glucagon, 10^{-5} M	315 ± 5	0 ± 1	343 ± 1	5 ± 3
NaF, 10^{-2} M	337 ± 8	67 ± 1	377 ± 10	78 ± 5
Methanol, 10%	336 ± 6	394 ± 12	52 ± 3	1 ± 3
Methanol, 20%	625 ± 7	725 ± 8	16 ± 1	2 ± 2
Ethanol, 10%	57 ± 1	0 ± 3	58 ± 2	1 ± 2
2-Propanol, 10%	78 ± 20	9 ± 2	59 ± 2	1 ± 3
Glycerol, 20%	570 ± 5	901 ± 10	33 ± 3	1 ± 2
Sorbitol, 2M	150 ± 6	420 ± 7	39 ± 2	18 ± 2

[a] The activity of adenylate cyclase was determined in the crude homogenate of rat liver under standard conditions stated in Table II. The final concentration of Lubrol PX was 2%. Average values from three experiments and standard deviations are given.

intact when the enzyme was bound to a solid carrier. It may be seen from Table VII (liver adenylate cyclase preparation bound to Sepharose) that the stimulation by sodium fluoride disappeared while the formation of the product remained intact or even increased in the presence of sorbitol.

All the above observations throw doubt on the assumption that the formation of the cyclic AMP on the one hand and of adenosine 5'-phosphate esters on the other hand is mediated by two different enzymes. On the contrary it cannot be excluded that both reactions are catalysed by the same enzyme. In the formation of the cyclic AMP, the transfer of the adenylyl residue is intramolecular while an intermolecular transfer takes place in the formation of adenosine 5'-phosphate esters. When the enzyme is bound in a complex on the membrane, the steric requirements of the cyclisation activity assert themselves and the enzyme responds to the specific activation with sodium fluoride and hormones. The intermolecular adenylyl transferase activity may be observed in the presence of higher concentrations of hydroxy derivatives which may change steric relations in the enzyme complex. When a catalytic unit is liberated from the complex by the action of detergents, the response to specific activators is lost and only the basal adenylyl transferase activity remains intact, manifesting itself in the formation of acyclic esters. The same behaviour is shown by the enzyme bound to Sepharose 4B. It is therefore of great importance in activity assays

TABLE VII

NaF and Sorbitol Stimulation of the Liver Adenylate Cyclase Preparation bound to Sepharose 4B (the activity is expressed in pmol/mg of the protein per 20 min)

Enzyme preparation[a]	Blank	NaF, 10^{-2}M	Sorbitol, 2M
Plasmatic membranes	12	300	231
Supernatant 100 000 *g* (in the presence of 0·4% Lubrol PX)	10	22	3 450
Supernatant bound to CNBr-Sepharose 4B	31	19	5 430
Supernatant bound to hexamethylenediamine $COCH_2Br$-Sepharose 4B	16	4	10 200

[a] Rat liver was homogenised in 75 mM Tris-HCl buffer solution in the presence of 12·5 mM $MgCl_2$. The crude homogenate was washed with three portions of the homogenisation mixture and subjected to centrifugations at 1 000 *g*. This fraction was regarded as plasmatic membranes. Their solubilisation was performed by rehomogenisation in the presence of 1% Lubrol PX; the final concentration of the detergent in adenylate cyclase assays was 0·4% and that of the other components as stated in Methods. The enzyme binding to Sepharose 4B was performed as described in the Experimental. The detergent was removed by repeated washings. Average values from two experiments are given.

of adenylate cyclase preparations to remove the simultaneously produced acyclic esters in cases when polyhydric alcohols are used as stabilizers of the enzyme or as solvents of the pharmaceuticals examined.

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