Les résultats du tableau 4 montrent encore que, d'une manière analogue à ce qui a été observé avec le groupe méthylène, les groupes méthyle de l'isopropyle sont aussi influencés par la nature du solvant et la température.

En particulier, la polarité du diméthylsulfoxyde tend à détruire le complexe de collision intramoléculaire au profit d'une association intermoléculaire dans laquelle les groupes méthyle sont magnétiquement équivalents.

Les auteurs remercient le *Fonds national suisse de la recherche scientifique* de l'aide financière qu'il a bien voulu accorder à ce travail.

Partie expérimentale

1. Les β hydroxyesters **1–41** ont été préparés d'après [1].

2. Tous les spectres ont été enregistrés par un spectromètre *Varian* A60A, à 37° et en solution dans $CDCl_3$ (concentration voisine de 0,2 M) sauf dans les cas indiqués précédemment.

Les spectres de système *ABX* avec couplage virtuel ont été calculés d'après le programme LAOCOON III [9] sur ordinateur IBM 1130.

Les déplacements chimiques sont exprimés en δ (ppm) et les constantes de couplage en Hz.

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185. Limitations of Affinity Chromatography: Solvolytic Detachment of Ligands from Polymeric Supports

by **Godefridus Ignatius Tesser**[1]), **Hans-Ulrich Fisch**[2])
and **Robert Schwyzer**

Institut für Molekularbiologie und Biophysik der Eidgenössischen Technischen Hochschule
CH-8049 Zürich

This paper is dedicated to Professor *Leonidas Zervas*, in respect and gratitude,
on the occasion of his recent 70th birthday.

(28. V. 74)

Summary. Ligands of the R—NH_2 type which are covalently bound to agarose, cellulose, or cross-linked dextranes by the conventional cyanogen bromide method are slowly detached from their supports by solvolytic processes occurring above pH 5 in aqueous surroundings. At pH values

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1) Present address: Dept. of Organic Chemistry, Catholic University of Nijmegen, Nijmegen, The Netherlands

2) Present address: Oberarzt, Psychiatrische Klinik Schlössli, CH-8618 Oetwil am See

between 5 and 10, the free ligands, R—NH_2, appear in solution. Above about pH 10, the carbamyl derivative of the ligand, $RNHCONH_2$, is the main product. Dilute ammonia releases iminocarbamyl derivatives, RNHC(=NH)NH_2. The latter type of compound has also been observed in tris-(hydroxymethyl)-aminomethane buffers at pH 8 and 9. Possible reaction mechanisms have been formulated on the basis of the structures suggested by *Axén & Ernback* for activated and substituted polysaccharides. Ligands attached to polyacrylamide gels (**A**) through amide bonds to carboxyl groups of the carrier, RNH—CO·**A**, are released at slower rates and invariably as the free, unsu b-stituted ligand molecules. The release reaction appear to be general and independent of the particular ligand structure. We therefore advise caution with all experiments that can be adversely influenced by the presence of free ligands (*e.g.* localization of hormone receptors on the cell surface). At pH5, sepharose-bound 8-(ε-aminocaproyl-β-aminoethylthio)-adenosine-3',5'-*cyclo*-monophosphate removes *cyclic* 3',5'-adenosine-monophosphate-binding proteins from their solutions, but the corresponding polyacrylamide derivative does not (pH 7).

Introduction. – The so-called 'immobilization' or 'insolubilization' of ligands by covalent attachment to polymeric supports has become a valuable experimental tool in many fields like enzymology and endocrinology [1]. However, as we have pointed out [2], the method can have its serious limitations because of a relatively facile release of ligands in aqueous surroundings. This is true not only for ligands attached to agarose, cellulose, and cross-linked dextranes by the usual cyanogen bromide method [3], but also for derivatives of polyacrylamide. The phenomenon was observed [2], because we were interested in a 'spacer' [1] derivative, **4**, of cyclic 3',5'-adenosine-monophosphate (cAMP) which, like the parent compound, is quantitatively detectable in low concentrations by its reaction with 'immobilized cAMP-binding proteins' [4]. After our first announcement [2], detachment of ligands has also been reported by others [5].

We raised the question of the chemical mechanism: Is the whole ligand detached as an entity by solvolysis (possibly facilitated by neighbour groups of the carrier gel such as hydroxyl or carboxyl), or does the cleavage occur predominantly within the spacer-ligand moiety? The work reported here points to a prevalence of the first type of reaction. This means that we are dealing with a generally neglected limitation of the usual techniques of ligand fixation. It might not be serious for large-scale affinity chromatography, but may be so in cases where it is essential to have absolutely no ligand in solution: For example, many results obtained with 'insolubilized' poly-

Table 1. *Compounds prepared for this study* [a])

1	Boc · εAca · $OC_6H_4NO_2(p)$	**10**	H_2N · CO—εAca—$NHC_6H_4NO_2(p)$
2	H · Aet—cAMP(8)	**11**	H_2N—C(=NH)—εAca—$NHC_6H_4NO_2(p)$
3	Boc · εAca—Aet—cAMP(8)	**12**	**S**—OC(=NH)—εAca—$NHC_6H_4NO_2(p)$
4	H · εAca—Aet—cAMP(8)	**13**	**A**—CO—εAca—$NHC_6H_4NO_2(p)$
5	**S**—OC(=NH)—εAca—Aet—cAMP(8)	**14**	Boc · εAca—εAca—$NHC_6H_4NO_2(p)$
6	**A**—CO—εAca—Aet—cAMP(8)	**15**	Boc · εAca—εAca—$NHC_6H_4NH_2(p)$
7	**S**—OC(=NH)—Ala(u—^{14}C)	**16**	Boc · εAca—εAca—NHC_6H_4NH—C(=NH)—O**S**
8	Boc · εAca—$NHC_6H_4NO_2(p)$	**17**	Boc · εAca—εAca—NHC_6H_4NH—CO—**A**
9	εAca—$NHC_6H_4NO_2(p)$		

[a]) For symbols and conventions see (7); εAca denotes ε-aminocaproic acid, Aet = 2-amino-ethanethiol-(1), cAMP(8) = cyclic 3',5'-adenosine-monophosphate with the substituent (sulfur of Aet) in position 8 of the purine ring (formulae see [2]), **S** = Sepharose (agarose), cellulose or cross-linked dextrane (Sephadex), **A** = polyacrylamide.

peptide hormones that were interpreted as indicating the outer cell membrane surface as the only site of hormone-target cell interaction may need further corroboration (see [2] for references).

In order to learn more about the chemistry of the detachment process, we decided to investigate in addition to the original ligand 8-(ε-aminocaproyl-β-aminoethylthio)-adenosine-3',5'-cyclo-phosphate, **4**, others with longer and shorter spacers, and with different 'prosthetic' groups, enabling easy detection. These ligands were linked to agarose, cellulose, and cross-linked dextrane type carriers (**S**) by the cyanogen-bromide technique [3], and to polyacrylamide gel (**A**) by amide bond formation with free carboxyl groups of the carrier according to [6]. The compounds are summarized in Table 1, their analytical data in Tables 2 and 3. The buffer systems used for the lytic reactions are indicated in Table 4.

Results. – 1) *Stability of 8-(ε-aminocaproyl-β-aminoethylthio)-adenosine-3',5'-*cyclo-*phosphate,* **4**. As the amide and thioether bonds (but not the 3',5'-cyclo-phosphate moiety, see [8]) might well be alkali-labile, we tested the stability of **4** in all buffer systems of Table 4. Not the slighest deterioration could be detected by thin-layer chromatography (TLC.) even after 83 days in 1 N ammonia at room temperature. Oxidation with *m*-chloro-perbenzoic acid or hydrogen peroxide led to extensive fragmentation within minutes (the products were not identified).

Table 2. *Results of elemental analyses*

No.	Mp. °C [a]	Composition	Mol. Weight (calc.)	% Calc./Found			
				C	H	N	P
1	116.5	$C_{17}H_{24}N_2O_6$	352.38	57.94 58.05	6.87 6.90	7.95 7.87	
2	170 †	$C_{12}H_{17}N_6O_6PS$	404.33	35.64 35.68	4.24 4.41	20.79 20.64	7.66 7.41
3	†	$C_{23}H_{36}N_7O_9PS$	617.61	44.73 44.63	5.88 5.90	15.88 15.97	5.02 4.84
4	†	$C_{18}H_{18}N_7O_7PS$	517.49 [b]	41.77 40.30 40.41	5.45 5.27 5.23	18.95 16.82 17.04	5.99 6.86 6.86
8	125	$C_{17}H_{25}N_3O_5$	351.39	58.10 57.97	7.17 7.15	11.96 11.90	
9	225	$C_{12}H_{18}N_3O_3Cl$	287.80	50.09 49.98	6.31 6.33	14.60 14.54	
10	217	$C_{13}H_{18}N_4O_4$	294.31	53.05 52.91	6.16 6.26	19.03 18.95	
11	221	$C_{13}H_{19}N_5O_3 \cdot 1/3(H_2SO_4)$	342.36	45.61 45.26	5.89 6.03	20.46 20.11	
14	120	$C_{23}H_{36}N_4O_6$	464.55	59.46 59.45	7.81 7.92	12.06 11.94	
15	62	$C_{23}H_{38}N_4O_4$ $(+0.5\ CH_3OH)$	450.59	62.64 62.53	8.95 8.88	12.43 12.10	

[a]) Decomposition is indicated by †.
[b]) Calc. with 4.40% ribose-3,5-*cyclo*-phosphate as contamination.

Table 3. *Chromatographic and optical data of cAMP derivatives*

Compound	Chromatographic data [R(F) × 100]					$[\alpha]^{25}_{\lambda(nm)}$ in 0.1N NaOH					
	PEC(C)	TC(C)	TS(C)	TC(D)	TA(D')	Conc. %	365	436	546	578	589
cAMP	33	63	67	43	–	–	–	–	–	–	–
Br—cAMP(8)	48	63	71	50	55	–	–	–	–	–	–
2	30	55	68	31	33	1.084	−302.8	−154.9	−81.7	−70.8	−67.7
3	63	70	78	66	64	1.165	−245.2	−123.0	−64.0	−55.2	−52.6
4	–	42	52	35	42	1.110	−280.0	−138.1	−71.4	−61.5	−58.2

Table 4. *Buffers and bases used for the release of 'immobilized' ligands from polymeric supports*

No.	Composition		pH
1	5×10^{-3} M	Sodium acetate + acetic acid[a])	5
2	–	Water alone	(5–6)
3	5×10^{-3} M	Imidazole/HCl[a])	6
4	5×10^{-3} M	Tris/HCl[a])	7
5	5×10^{-3} M	Tris/HCl[a])	8
6	0.08 M	Triethylamine/AcOH	8.5
7	5×10^{-3} M	Tris	9
8	0.08 M	Triethylamine	11.5
9	1 M	Ammonia	High

[a]) containing NaCl to give 15×10^{-3} M.

2) *Instability of 'immobilized' alanine*, **7**, *and 'immobilized' εAca-Aet-cAMP(8)*, **5**, **6**. It was found that water, sodium acetate, imidazole, and tris-(hydroxymethyl)-aminomethane buffers release alanine and εAca-Aet-cAMP(8), **4**, in unchanged form from **7**, **5**, and **6** (for changes, see below). This was shown by TLC. directly and after acetylation of the products of **5** and **6** with *p*-nitrophenyl-1-^{14}C-acetate which reacts readily with the aliphatic amino groups of the εAca-end of **4**, but not with the amino and hydroxyl groups of the adenosine moiety. Fig. 1 shows that the triethylamine-acetic acid buffer, pH 8.5 (6, Table 4), releases predominantly the free εAca-Aet-cAMP(8), **4**, from **5** (**6** gives similar results).

A quantitative study with the assay of *Fisch, Pliška & Schwyzer* [4] which detects all the cAMP derivatives that might be released from **5** and **6** gave the results shown in Fig. 2a and 3. The agarose, cellulose and crosslinked dextrane carriers behaved in much the same manner, being most stable at pH 5, and losing their ligands more rapidly as the pH is raised (Fig. 2a). On the other hand, the polyacrylamide derivative, **6**, was most stable at pH 7, suggesting general acid and base catalysis of the hydrolytic ligand cleavage (Fig. 3). 'Immobilized' alanine, **7**, behaved very similarly, although the velocities are roughly only half those of **5** (Fig. 2b).

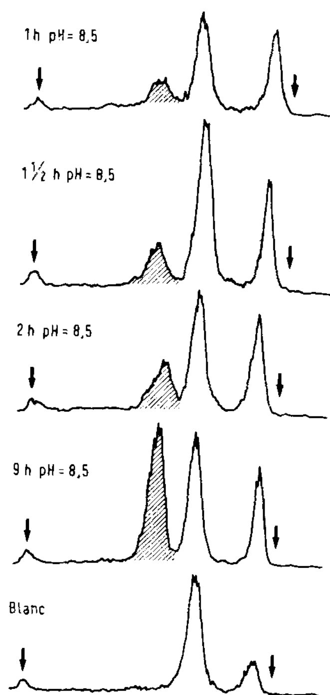

Fig. 1. *Release of* **4**, *εAca—Aet—cAMP(8), from its Sepharose conjugate*, **5**, *during different time intervals at pH 8.5 and 4°.* Equal amounts of supernatant were treated with *p*-nitrophenyl-1-^{14}C-acetate, and the products separated by TC (D). Start at left, solvent front at right arrow (abscissa). peaks indicate radioactivity in arbitrary units (ordinate). Shaded areas correspond to 1-^{14}C—Ac · εAca—Aet—cAMP(8)

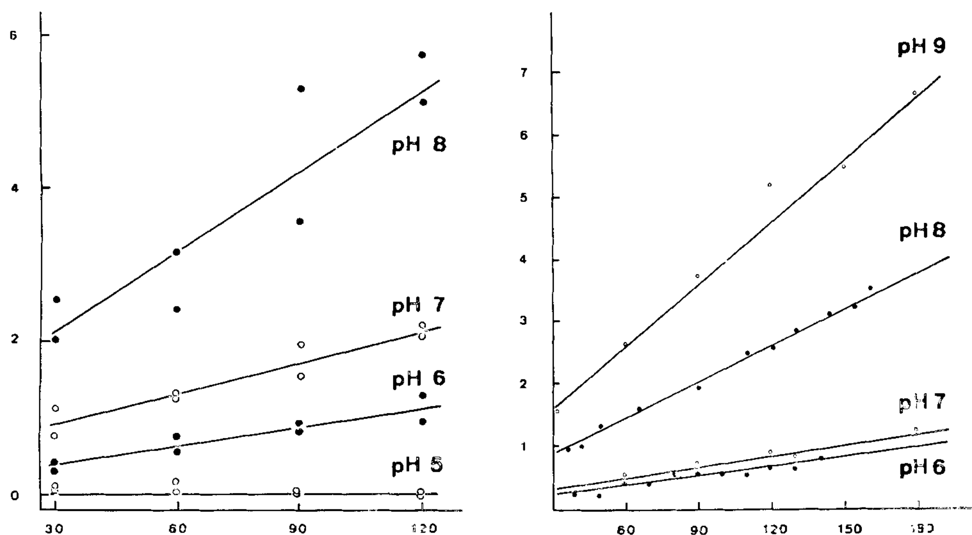

Fig. 2. *Ligand release from 1 g of moist, substituted Sepharose gels* **5** *and* **7**, *at 4°: pH and time dependence*. Ordinates: nanomol of ligand found in solution. Abscissae: time in minutes. Ligands: **4**, ε—Aca—Aet—cAMP(*8*), total substitution about 300 nanomol per gram (left, a); u-^{14}C-alanine, about 1000 nanomol per gram (right, b).

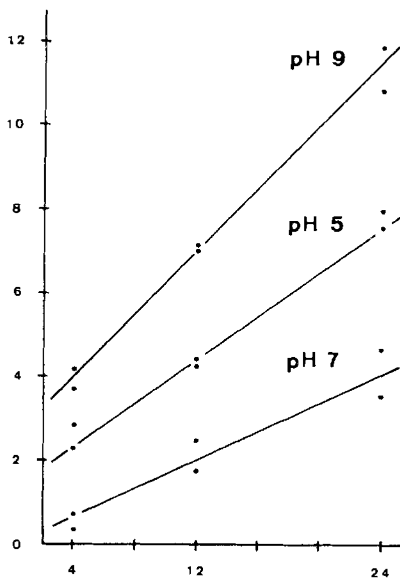

Fig. 3. *Release of* **4**, *εAca—Aet—cAMP(8), from 1g of moist, substituted polyacrylamide gel*, **6**, *at 4°: pH and time dependence*. Total substitution about 3000 nanomol per g. Ordinates: nanomol of **4** released; abscissae: time in hours.

3) *Chemical alterations of the ligands during their cleavage from* **5**, **7**, **12**, *and* **13**. The treatment of **5** (agarose, cellulose, and cross-linked dextrane) with the tris-buffers at pH 8 and 9 (nos. 5 and 7, Table 4) and especially with triethylamine, pH 11.5 (no. 8) and ammonia (no. 9) released εAca-Aet-cAMP(8), **4**, in forms unreactive towards *p*-nitrophenyl-1-^{14}C-acetate. A first indication of the possible nature of the products was obtained from experiments with 'immobilized' ^{14}C-alanine, **7**. In the tris-buffers pH 8 and 9, 90% and 40%, respectively, of the total radioactivity released were due to free alanine, whereas another compound which moved more slowly on TLC. amounted to 10% and 60%. The slowly-moving spot was tentatively identified as a guanidino compound (positive *Sakaguchi* test). Similarly triethylamine (no. 8) released two new compounds, X^1 and X^2 (see experimental part) which are believed to be carbamoyl-alanine and 4-methyl-hydantoin.

To clarify the situation, we examined the readily available *p*-nitroanilide, **12**, obtained by coupling of εAca-$NHC_6H_4NO_2$, **9**, with cyanogen bromide-activated Sepharose. Ammonia (no. 9) released almost exclusively ε-guanidinocaproic acid *p*-nitroanilide, **11**. Triethylamine (no. 8) gave rise to mainly ε-carbamylaminocaproic acid *p*-nitroanilide, **10**, accompanied by small amounts of *p*-nitroaniline and ε-carbamoylaminocaproic acid as secondary products of hydrolysis. Triethylamine-acetic acid buffer, pH 8.5 (no. 6), finally, produced ε-aminocaproic acid *p*-nitroanilide, **9**, as the only product. All products were identified by comparative TLC., **9** also as its ^{14}C-acetyl-derivative.

The polyacrylamide derivative, **13**, proved to be more stable under all conditions (*cf.* Fig. 3) and to release exclusively the complete, free ligand, **9**.

Test experiments showed that the modified gels **16** and **17**, containing Boc · εAca–εAca–$NHC_6H_4NH_2$, **15**, behaved similarly.

4) *Retention of adrenal cAMP-binding protein by* **5** *and* **6**. Insolubilized adrenal cAMP-binding protein binds εAca–Aet–cAMP(8) reversibly ($K^{ass} \simeq 2.3 \cdot 10^8 M^{-1}$), and the association constant isn't strongly influenced by pH variation in the neutral to weakly acidic and alkaline region [2] [6]. Because **5** is stable at pH 5, we decided to see whether it would bind soluble adrenal cAMP-binding protein at this pH. By careful agitation of a protein sample with a 10^4 molar excess of freshly washed **5** at pH 5 and 4° for 15 minutes, the binding capacity for ^{3}H-cAMP of the supernatant decreased from 16.5 to 2.1 nmol per liter (assay according to [9]). This means that about 90% of the binding protein are removed from the solution by this 'batch-wise' procedure. On the other hand, the polyacrylamide gel derivative, **6**, did not bind any appreciable amounts at pH 7.

Conclusions. – The results of this study suggest that the release of ligands from their carrier gels occurs predominantly at the point of fixation to the matrix and might be facilitated by anchimeric assistance of the neighbouring carboxyl and carboxamide groups in polyacrylamide gels, and hydroxyl groups in agarose, cellulose, and cross-linked dextranes.

Ligand cleavage from polyacrylamide supports can be compared with the facile solvolysis of the native amide groups which was explained by anchimeric assistance [10].

Based on the work of *Axén & Ernback* [3] who postulated a cyclic iminocarbonate [–O–C(=NH)–O–] as a reactive form of polysaccharide – type gels activated by cyanogen bromide, we have devised the tentative scheme of Fig. 4 to explain our findings with **S**-bound ligands. The top portion delineates the activation process, the attachment of a ligand, RNH_2, and hydrolysis according to [3] (somewhat simplified). The lower part shows the reactions assumed to take place: Hydrolysis between pH 5 and 10 might operate through protonation of the strongest base, RNH-, releasing the free ligand. Above pH 10, RNH- would be unprotonated, and the oxide oxygen atoms would become the best leaving groups, producing the carbamoyl derivative. A similar situation prevails in ammonia with the difference that now ammonia would strongly compete with hydroxyl as a nucleophile, giving a substituted guanidine. Both observed substitution reactions support the structures suggested in [3] for the covalently bound ligand, –O–C(=NH)–NHR and its cyclic form (Fig. 4, top).

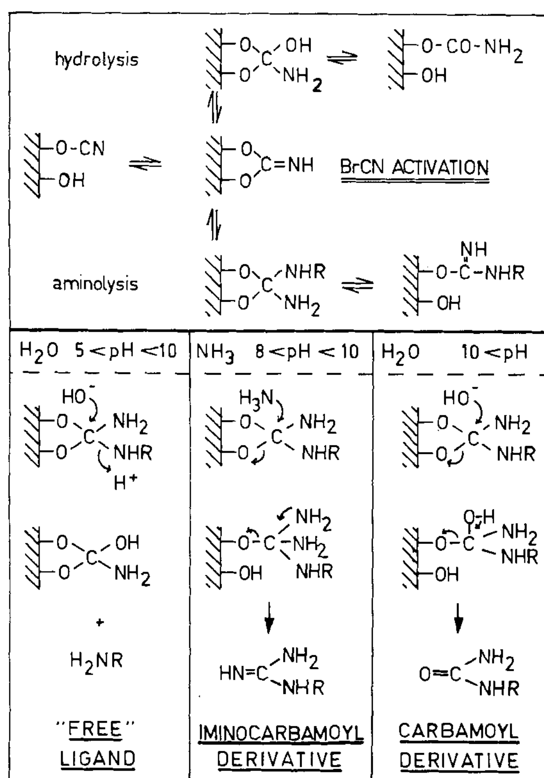

Fig. 4. *Suggested mechanisms for cyanogen bromide activation, amine substitution* (3), *and ligand release* (this study)

As might have been expected, the treatment of Boc · εAca–Aet–cAMP(8), **3**, with 90% trifluoroacetic acid leads to considerable hydrolysis of the purine riboside bond. The produced ribose-3,5-*cyclo*-phosphate was found to contaminate the analytical sample of **4**, εAca–Aet–cAMP(8).

It is an interesting, although unexplained fact that **5**, **S**–OC(=NH)–εAca–Aet–cAMP(8), appears to be well suited for affinity chromatography of cAMP-binding proteins, whereas **6**, **A**–CO–εAca–Aet–cAMP(8), is not. This clearly demonstrates the highly empiric state of affairs in this important field. It also illuminates some of the difficulties encountered in the related technique of solid-phase peptide synthesis.

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Experimental Part

M.p. were determined in open capillary tubes and are uncorrected. *Thin-layer chromatograms* (TLC.) were run on alumina +12% gypsum (TA), silica gel with a fluorescence indicator (*Merck*; TS), cellulose with a fluorescence indicator (*Merck*; TC), or cellulose impregnated with polyethylene-imine (PEC), and the product spots made visible by conventional methods (fluorescence quenching, ninhydrin, *Sakaguchi*, and *Reindel-Hoppe* reagents). The Rf-values are given by numbers followed by capital letters indicating the solvent systems: (A) $CHCl_3$/MeOH, 4:1; (B) 1-BuOH/AcOH/H_2O, 10:1:3; (C) 2-PrOH/conc. NH_3/H_2O, 7:1:2; (D) 1-BuOH/pyridine/AcOH/H_2O, 42:24:4:30; (D') same, but 38:24:8:30; (E) $CHCl_3$/MeOH/H_2O, 4:4:2; (F) MeOH/H_2O, 7:3; (G) 1-BuOH/AcOH/H_2O, 67:10:23 volume ratios).

Polymeric supports. In most experiments agarose (Sepharose 2B and 4B, *Pharmacia*, Uppsala, Sweden) was used. The results were invariably the same with crosslinked dextrane (Sephadex G-10, *Pharmacia*) and cellulose (*Whatman*): therefore only those obtained for sepharose shall be recorded in detail. Activation by the cyanogen bromide method was carried out according to (3). Purchased activated materials (*Pharmacia*) gave indistinguishable results. In experiments with polyacrylamide, Biogel P-100 (*Bio-Rad*, Richmond, Cal., USA) was used. It was partially hydrazinolysed and activated by treatment with HNO_2 according to *Inman & Dintzis* [6].

Ligands. Cyclic 3',5'-adenosine monophosphate (cAMP) was purchased from *Fluka* (Buchs, Switzerland). Ubiquitously labelled ^{14}C-L-alanine (162 Ci/mol) and 1-^{14}C-acetic anhydride (118 Ci/mol; 5 per cent (w/w) solution in benzene) were obtained from *The Radiochemical Centre* (Amersham, England).

Enriched cAMP binding protein was prepared essentially according to *Brown et al.* [9] as described by *Fisch et al.* [4] and used as solutions of 1 mg protein per ml. The binding capacity of these solutions was 16.5 nmol (3)H-cAMP per l. The preparations contained a relatively high amount (12.4 nmol/l) of cAMP, most probably complexed by an equivalent quantity of binding protein (in addition to the 'free', reactive 16.5 nano-equivalents).

Total 'soluble cAMP activity' released from polymeric supports was assayed by the method of *Fisch et al.* (4). Its components were characterized by TLC. comparison with synthetic materials. 8-(ε-aminocaproyl-β-aminoethylthio)-cAMP, **4**, was detected as the N(ω)-acetyl derivative after reaction with *p*-nitrophenyl-1-^{14}C-acetate of the crude residues obtained by evaporation of the aqueous filtrates.

p-*Nitrophenyl*-t-*butoxycarbonyl-ε-aminocaproate* (**1**). 6-Aminohexanoic acid was condensed with *t*-butyl-azidoformate according to the general method of *Schnabel* [11]. Low melting solid, m.p. 39.5°; 90% yield. This compound (11.56 g = 50 mmol) and *p*-nitrophenol (8.34 g = 60 mmol) were dissolved in 50 ml of ethyl acetate. The mixture was cooled to 0° and treated with dicyclohexyl-carbodiimide (10.3 g = 50 mmol). After reaction for 30 min at 0° and for 1 h at RT.[1]), the solution was filtered from the precipitated dicyclohexyl-urea and then concentrated *in vacuo* to a thick sirup. This was triturated with 35 ml of isopropyl-alcohol which caused the crystallization of the desired product (19.3 g = 91%). Recrystallization from toluene (1 g in 3 ml) yielded pure, pale yellow crystals (15.44 g = 73%; TS: 0.72 (A), 0.79 (D).

8-(β-aminoethylthio)-adenosine-3',5'-cyclo-*phosphate* (**2**). 8-Bromo-adenosine-3',5'-*cyclo*-phosphate (12) (1.027 g = 2.5 mmol) was suspended by stirring in 5 ml of methanol contained in a 25 ml flask. Cysteamine hydrochloride (1.204 g = 10.7 mmol) was added, and the flask was filled to the brim with 20 ml of 1M methanolic sodium methoxide. The flask was stoppered with exclusion of air and its contents were stirred for 15 h at RT. The insoluble sodium halides were removed

[1]) RT. = room temperature.

by filtration and washed with a small amount of methanol. Filtrate evaporation left a thick sirup which was diluted with 15 ml of water. The solution was filtered through a Millipore filter and acidified with HCl to pH = 5. The zwitterionic nucleotide derivative, **2**, precipitated as fine, blade-like crystals. They were collected after cooling and dried, whereupon they lost crystal solvent and were transformed to a white powder (94.5% yield). Recrystallization from water gave chromatographically pure material without a definite m.p. (carbonization at 170°). Its NMR. spectrum agreed with that expected for the structure **2**. For chromatographic data and optical rotation, *cf.* Table 3.

*8-(*t-*Butoxycarbonyl-ε-aminocaproyl-β-aminoethylthio)-adenosine-3′,5′-*cyclo-*phosphate* (**3**). Compound **2** (1.013 g = 2.5 mmol) were suspended by stirring in 4 ml of purified dimethylformamide; **1** (1.06 g = 3 mmol) and triethylamine (0.37 ml = 2.6 mmol) were added. The suspended nucleotide rapidly disappeared into solution, and after about 2 min no more starting material could be detected chromatographically. After 15 min 15 ml each of toluene and water were added, and the yellow colour discharged with acetic acid. The upper layer was removed, and the lower one extracted 5 times with fresh toluene. The organic extracts were discarded and the aqueous phase was concentrated *in vacuo* after cooling and acidification to pH = 2. The product, **3**, was precipitated as a voluminous gel by trituration of the residue with acetone. Filtration, washing with peroxide-free ether, and drying yielded 1.226 g (79.5%) of chromatographically pure **3**. The analytical sample was obtained as follows: 100 mg of crude **3** were dissolved in 500 μl of 2N ammonia; this solution was diluted with 500 μl ot water, cooled, and treated with 500 μl of 2N HCl-solution. The colourless precipitate that formed after neutralization was filtered, washed with 1 ml of ice-cold water, and dried. Only 47 mg were recovered. The NMR. spectrum agreed with structure **3**.

*8-(ε-Aminocaproyl-β-aminoethylthio)-adenosine-3′,5′-*cyclo-*phosphate* (**4**). Trifluoro acetic acid and water were mixed in the volume proportion of 9:1. Nitrogen was bubbled through during 15 min, and 20 ml of this reagent were introduced into a flask filled with nitrogen and containing 1.004 g of **3**. The compound dissolved quickly with evolution of CO_2. After 15 min, the solution was added dropwise to 250 ml of peroxide-free ether (vigorous stirring!). The suspension was cooled and the precipitate gathered by filtration, washed with ether, and dried over moist sodium hydroxide pellets. The dried compound was dissolved in a small amount of methanol, giving a weakly acidic solution (apparent pH ≃ 3). Insoluble material (14 mg) was removed by filtration: it was rapidly discoloured on contact with air. The filtrate was diluted with ether. At first, a reddish-brown oil precipitated. The supernatant solution was decanted and added dropwise into 50 ml of ether. A perfectly white precipitate formed (548 mg = 65.2%). The oily fraction contained, apart from **4**, a minor constituent of lower polarity (TS); it was discarded. The main product seemed pure in each of the solvent systems used (table 3), as the detection was directed to the revelation of the purine moiety. It was used as such. On account of its phosphorus content the presence of a small amount (4.40%) of *cyclic*-3,5-ribose phosphate became manifest.

Compound **4** exhibited excellent stability towards the volatile alkaline buffers of Table 4; a sample was dissolved in 1N ammonia, kept at RT. for 83 days, and intermittently checked by TLC. (Table 3) and high voltage electrophoresis on cellulose (pH = 1.9). Not the slightest deterioration was observed.

Covalent attachment of **4** *to agarose (cellulose and dextrane) supports* (**5**). Sepharose 4 B activated with BrCN (11.3 g) was washed free of BrCN and salts, and rinsed with 10 portions, 25 ml each, of borate buffer (0.025M, pH = 10.2), precooled to 0°. After the last wash, it was sucked dry for 2 sec. and quickly added to a solution of **4** (33 mg) in 5 ml of the same cold buffer. The vessel was slowly rotated axially in a tilted position for 19 h at RT. (20°). The contents were then diluted with fresh buffer and poured into a chromatographic tube with a sintered glass bottom. The gel was washed with a total of 250 ml of the above borate buffer; after passage of 50 ml, the optical density at 260 nm had already fallen to very low values. Subsequent washing was achieved with 500 ml 0.2N sodium hydrogencarbonate/0.2N NaCl buffer, followed by 500 ml 0.02M tris-buffer, pH = 7.4. Finally, the gel was washed for 15 h with, and then stored until required in 0.02M sodium acetate buffer, pH = 5. As estimated from the nucleotide content of the first washes, the gel contained a total of 3.3 μmol of 4 per ml, using a molar extinction coefficient of 13,200 at 260 nm.

Covalent attachment of **4** *to polyacrylamide gel* (**6**). The activated gel containing carboxylic acid azide groups, prepared from 500 mg of lyophilized hydrazide, was washed free of inorganic salts with water and 0.1 M N-methylmorpholine (pH $\simeq$ 8–9) and then transferred to a Nalgene reaction flask containing 53 mg of **4**, dissolved in 5.3 ml of N-methylmorpholine. After 3 h of gentle agitation by axial rotation, a dilute solution of ammonia containing 500 mg of ammonium acetate (pH = 9; 5.5 ml) was added in order to convert unreacted carbonic acid azide to amide groups. The resin was filtered and thoroughly washed with water after a reaction time of 16 h. Judging from the optical density of the first filtrates, an upper substitution limit of about 3.7 μmol of **4** per g of fully swollen gel (total weight: 16.4 g) was calculated.

It proved to be impossible to achieve quantitative hydrolytic detachment of the nucleotide from the carrier without partial destruction of the purine system. Equal volumes of gel and 12 N HCl kept at RT. for 20 h liberated only negligible amounts of material with the spectrum of the adenine chromophore. Under conditions of protein hydrolysis (6 N HCl, *in vacuo*, under N_2, 105°, 24 h) only brown decomposition products were observed. When the gel (1 g) was left for one week at RT. with 1 ml of 2 N ammonia, 50 μl of the supernatant contained enough of the ligand to allow chemical detection on thin layer plates. The nucleotide was released as the complete and unaltered molecule, **4**.

Covalent attachment of u-^{14}C-alanine to agarose (**7**). 250 μCi (148 μg = 1.53 μmol) of uniformly labelled ^{14}C-L-alanine were added to one gram of freshly activated sepharose 4B, mixed with 500 μl of borate buffer (0.025 M, pH =10.2). The suspension was agitated by axial rotation at 4° overnight. The gel was washed successively with 500 ml portions of water, 10^{-3} N hydrochlorid acid, and 1 N sodium chloride solution, and then stored in cold acetate buffer (0.05 M, pH 5, 4°). Its specific activity was determined by scintillation counting of samples completely dissolved in 6 N hydrochloric acid and found to be 195 μCi (= 1.2 μmol) per gram. With this preparation, loss of alanine or its derivatives corresponded to 355 dpm of radioactivity released per picomol.

t-*Butoxycarbonyl-ε-aminocaproic acid* p-*nitroanilide* (**8**). *t*-Butoxycarbonyl-ε-aminocaproic acid (1.4 g = 6.05 mmol) and *p*-nitroaniline (0.85 g = 6.15 mmol) were dissolved in 5 ml of dry pyridine. The solution was cooled to –10°, and 0.5 ml of $POCl_3$ were added dropwise with stirring. The stoppered flask was kept for 30 min in the refrigerator. The solution was diluted with 100 ml of water, and the precipitated product (1.9 g) was recrystallized from ethyl acetate: 1.2 g (= 63%) of analytically pure 8; TS: 0.70 (A), 0.87 (B), 0.83 (C).

ε-Aminocaproic acid p-*nitroanilide hydrochloride* (**9**). 3.12 g of Boc · Aca · NH · C_6H_4 · NO_2(*p*) (**8**) were dissolved in a mixture of methanol (4 ml) and ethyl acetate (2 ml). After addition of 12 ml of a 2 M solution of dry HCl in ethyl acetate, crystallization of the product commenced after about 5 min and was essentially complete after 3 h in the refrigerator (2.41 g = 94%). Recrystallization by dilution of a 10 per cent solution in methanol with an equal volume of dry ether. TS: 0.35 (B), 0.23 (C).

ε-Carbamoylaminocaproic acid p-*nitroanilide* (**10**). Preparation in analogy to that of ε-carbamoylaminocaproic acid (13) as follows: **9** (288 mg = 1 mmol) was dissolved in water (5 ml) with potassium cyanate (162 mg = 2 mmol). In a few minutes, 266 mg (= 88%) of crude, solid **10** had separated. Recrystallization from hot 90% acetic acid gave 160 mg of pure **10**, m.p. 217°. Homogeneous on TS: 0.75 (C), 0.68 (G), as compared with 0.29 (C) and 0.36 (G) for 9. Ninhydrin negative (9 is positive!), detection by fluorescence quenching and the *Reindel-Hoppe* reaction. – Typical IR. (cm^{-1}): 3490, 3390 (amide NH), 1660, 1500 (amide I and II), 1605, 1500 (phenyl H), 1540, 1340 (arom. NO_2), 1260 (N–O). – UV. (max.): 315 nm, ε(molar) = 11,600.

ε-Guanidinocaproic acid p-*nitroanilide sulfate* (**11**). **9** (288 mg = 1 mmol) and S-methyl isothiouronium sulfate (280 mg = 1 mmol) were dissolved in water (5 ml) by gentle heating. To the yellow solution triethylamine (0.42 mol = 3 mmol) were added at once. The now turbid solution was vigorously shaken and carefully heated until it became clear. After cooling, an oil which rapidly crystallized separated out. This product was recrystallized from boiling water, giving **11** (225 mg = 66%), m.p. 221°. Homogeneous on TS: 0.16 (C), 0.45 (G). Detection by fluorescence quenching, *Reindel-Hoppe*, *Sakaguchi* (all positive), ninhydrin negative. – IR. (relevant bands cm^{-1}): 3500–3000 (guanidinium NH), 1665, 1500 (amide I, II), 1600, 1500 (phenyl H), 1560, 1340 (arom. NO_2), 1120–1070 (SO_4^{-2}). – UV. (max.): 315 nm, ε(molar) = 13,500.

t-*Butoxycarbonyl-ε-aminocaproyl-ε-aminocaproic acid* p-*nitroanilide* (**14**). **9** (2.406 g = 8.36 mmol) and **1** (2.82 g = 8.00 mmol) were slurried in dimethylformamide (10 ml), and the solution heated

to 50°. Then, at that temperature, triethylamine (1.2 ml = 8.6 mmol) were added and lumps of starting material crushed with a glass rod. The reaction mixture was agitated overnight at RT. and then poured into water whereupon a rapidly solidifying oil was precipitated. Filtration afforded crude **14** which was washed with water and diisopropyl ether. Crystallization from ethyl acetate: colourless crystals (3.52 g = 94.8%); TS: 0.73 (A), 0.76 (B). – IR. (relevant bands, cm^{-1}, KBr): 1710, 1680 and 1650 (carbonyl), 1330, 1250 and 1104 (nitro), 1160 (*t*-butyl).

t-*Butoxycarbonyl-ε-aminocaproyl-ε-aminocaproic acid* p-*aminoanilide* (**15**). **14** (1 g) was dissolved in methanol (20 ml) and hydrogenated in the presence of 50 mg of Pd/C (10%). Hydrogenation was complete after about six h. The product, **15**, was isolated by filtration of the solution and evaporation of the solvent. It crystallized upon addition of ethyl acetate and was purified by recrystallization from methanol/ethyl acetate: 753 mg = 81%. TS: 0.62 (A), 0.59 (B). – IR. absorption like that of **14**, but lacking the frequencies due to the nitro group. For elemental analysis, **15** was dried over P_2O_5 at 20° for 15 h, however, it still retained a small quantity of solvent (methanol?). – UV. (max.): 260 nm, ε(molar) = 16,000.

Covalent attachment of anilides to agarose and polyacrylamide gels (**12**, **13**, **16**, **17**). Using procedures similar to those for the preparation of **5**, **6** and **7**, both **9** and **16** were conjugated with activated agarose and polyacrylamide gels, respectively. The resins were found to contain between 3 and 7 μmol of ligand per ml of swollen gel by the spectroscopic difference method described above.

Detection of unmodified ligands among the products released in buffer suspensions. Samples (about 500 mg) of swollen gels, washed until the filtrates contained no more detectable 'free ligands', were mixed with 2.5 ml each of a buffer or base solution according to Table 4. The suspensions were slowly rotated at 4°, taking care not to damage the grain texture mechanically. At suitable time intervals (1–24 h) the gels were spinned down or filtered, and 10 μl aliquots of the absolutely clear filtrates or supernatants were withdrawn, transferred to polyethylene vials and evaporated in a vacuum desiccator. The usually invisible residue was dissolved in 10 μl of dimethylformamide containing 10% (v/v) of triethylamine. A benzene solution of $1\text{-}^{14}C$-acetic acid *p*-nitrophenylester (10 μl, 1 μCi) was added, and the mixtures kept for 1 h at 37° and again evaporated *in vacuo*. The residues were dissolved in 10 μl of methanol and applied to TS (anilides) or TC ('cAMP') plates. After development, the chromatograms were scanned and the positions of the radioactive zones compared with those of authentic samples (non radioactive materials, **4** and the anilides, were pretreated as above with *p*-nitrophenyl $1\text{-}^{14}C$-acetate).

Detection of modified ligands among the products released in buffer solutions. (i) *Ammonia.* Swollen samples (100 mg) of agarose gels containing ε-aminocaproyl-*p*-nitroaniline as ligand were mixed with 2 N ammonia (100 μl) and left for 48 h at RT. After centrifugation, the supernatant was removed and evaporated. Addition of ammonium sulfate solution precipitated a straw-yellow compound which appeared on TS as a strongly fluorescence-quenching spot, giving a positive *Sakaguchi* reaction. Its position coincided sharply with that for authentic ε-guanidinocaproyl-*p*-nitroanilide, **11**. The compound did not react with *p*-nitrophenyl-$1\text{-}^{14}C$-acetate. (ii) *Triethylamine* Using a similar procedure, the samples were treated with 1% triethylamine solution. In the supernatant, ε-carbamylaminocaproyl-*p*-nitroaniline, **10**, was detected, accompanied by *p*-nitroaniline and ε-carbamoylamino caproic acid. An authentic sample of **10** (12) revealed the same spots on treatment with the base. No reaction with *p*-nitrophenyl-$1\text{-}^{14}C$-acetate was observable. (iii) *Triethylammonium acetate* (*pH = 8.5*). Similar tests with this reagent produced no guanidinium and only traces of the carbamoyl compound. The major product was the free, unaltered ligand, **10**, which was readily acetylated. (iv) *Polyacrylamide gels* expelled their ligands under the same conditions (i–iii) as complete, underivatized compounds. Secondary hydrolysis of the acid nitroanilide bond, however, occurred in the ammonia and triethylamine solutions. (v) *Agarose gels containing the alanine ligand* were treated for 4 h with triethylamine solution as above. They released no alanine, but carbamoylalanine, instead: two unidentified, minor spots with less polar character (X_1, X_2) were also observed. Two products corresponding to X_1 and X_2 were detected in the crude reaction product obtained by treating authentic carbamoylalanine with mineral acid under the conditions for ring closure to 4-methyl-hydantoin. The products were identified by TLC:

	Alanine	Carbamoylalanine	X_1	X_2
TS (E)	0.51	0.61	0.74	0.91
TS (F)	0.66	0.83		

Reaction of adrenal cAMP-binding protein with immobilized ligand **4**. All operations were carried out at 4°. Into two tubes, A and B, equal amounts (500 mg) of well washed **5** were placed. Alternatively, the polyacrylamide gel, **6**, was used in *tris-HCl* buffer at pH 7. The gels had been pretreated and well washed with 0.01M acetate buffer, pH 5, containing 15 mmol of NaCl. Into A and another, empty tube C, 5 ml of the crude binding protein preparation, dialyzed against the same buffer, plus 0.5 ml of the buffer, were pipetted. To the gel sample in B, only 5 ml of the buffer were added. The tubes were closed, and their contents carefully mixed for 15 min. After centrifugation, the supernatants from A, B, and C were collected. Supernatants A and C were divided into two parts each, one of which was used for the determination of cAMP-binding capacity according to *Brown et al.* (8), the other served – following deproteinization with trichloroacetic acid and careful ether extraction – for the determination of 'soluble cAMP activity' (4). Supernatant B was used directly in the latter test.

p-*Nitrophenyl-1-^{14}C-acetate*. 1-^{14}C-acetic anhydride (500 μCi, 4.03 μmol) was used as a benzene solution (5%, v/v) in an ampule equipped with a ground-joint conus. The synthesis was performed with a bridge-shaped apparatus that consisted of a 2 mm inner-diameter glass tube, approximately 10 cm long, carrying a sealed-on three-way disk vacuum valve in the middle. At both ends two ground jackets fitting the conus of the ampule were sealed on in a direction perpendicular to the main tube and antiparallel to the opening of the valve. A capillary tube 5 cm long and with 3 mm inner diameter, carrying a ground-joint conus, served as the reaction vessel.

To 504 mg (3.63 mmol) of *p*-nitrophenol (analytical grade) 24 μl of pure, dry pyridine (8 mol %) were added, and the mixture shaken for 2 days. Then, 0.530 mg of this mixture (3.63 μmol, 90% of the theoretical amount) were carefully weighed into the reaction vessel, a steel ball of 1 mm diameter was added, and the vessel was connected to the bridge. The ampule containing the anhydride was opened and also attached; both vessels were cooled with liquid N_2. After evacuation, the three-way valve was turned to exclude the pump and connect the two vessels. The one with the anhydride was carefully warmed with a current of air, while the reaction vessel was kept in the liquid N_2. After distillation of all of the anhydride, dry air was allowed to enter the apparatus, and cooling was interrupted. The reaction mixture was agitated with a magnet for 30 min at RT. Then the distillation process was carried out as above in the reverse direction. To complete the removal of the solvents, the reaction vessel was heated to 80°. The residue was dissolved in 2 ml of dry benzene and stored in a glass counting vial. The solution contained 0.135 μCi per μl; it obviously was contaminated with some radioactive acetic acid. As this could cause no harm in the further reactions, the solution was used without further purification.

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