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PAPER CHROMATOGRAPHY COMBINED WITH PHOSPHODIESTERASE TREATMENT IN THE DETERMINATION OF RADIOACTIVELY LABELLED CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE

JAN-BJØRN OSNES, THORALF CHRISTOFFERSEN, JØRG MØRLAND AND IVAR ØYE
Institute of Pharmacology, University of Oslo (Norway)

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SUMMARY

The measurement of radioactively labelled cyclic 3',5'-adenosine monophosphate formed from labelled ATP in whole cells or coarse tissue particle preparations requires complete removal of labelled metabolites. Some of these metabolites occur in concentrations several orders of magnitude above that of cyclic 3',5'-adenosine monophosphate and some of them are difficult to separate from cyclic 3',5'-adenosine monophosphate by simple chromatographic procedures. The method presented has proved itself suitable for measuring the amount of [^{14}C]cyclic 3',5'-adenosine monophosphate in samples rich in other ^{14}C-labelled metabolites. The procedure consists of paper chromatography, subsequent enzymatic conversion of cyclic 3',5'-adenosine monophosphate to adenosine 5'-phosphate (AMP) and then re-chromatography in the same system. The AMP thus obtained originates from cyclic 3'-5'-adenosine monophosphate only, and the radioactivity of AMP is measured to calculate the amount of cyclic 3',5'-adenosine monophosphate.

INTRODUCTION

The level of cyclic 3',5'-adenosine monophosphate (cAMP) in biological material usually amounts to less than 0.1 % of the adenine nucleotide pool, and the apparent activity of adenyl cyclase is small compared to the apparent activity of ATPases and other enzymes acting on adenine nucleotides and adenine nucleotide metabolites[1–3]. In recent years numerous studies on the biosynthesis of cAMP from radioactively labelled ATP have been carried out by adding labelled ATP to broken cell preparations[3–26] or by labelling the endogenous ATP pool by exposing cells or tissue preparations to labelled adenine or labelled adenosine[27–30]. In both cases the total amount of labelled metabolites formed exceeds the amount of labelled cAMP by several orders of magnitude, and the separation of these metabolites from the labelled cAMP fraction before counting its radioactivity is of course critical in this type of study[3,6,9–11].

The method presented below, now routinely used in our laboratory, has proved itself particularly suited for measuring the amount of ^{14}C-labelled cAMP in samples

rich in labelled metabolites, which tend to co-chromatograph with cAMP[31]. On developing this method a rat liver preparation (which is rich in ATPases and other purine-metabolizing enzyme systems) was used as a source of adenyl cyclase.

MATERIALS AND METHODS

Chemicals

The following chemicals were used: [8-^{14}C]ATP and [2-^{3}H]AMP (The Radiochemical Center, Amersham), [^{3}H]cAMP (Schwarz Bio-Research, Inc.), non-radioactive purine derivatives (Sigma Chemical Co.), 2,5-bis-[5′-*t*.-butyl-benzoxazolyl (2′)]-thiophene (BBOT) (Ciba Ltd.) and beef heart 3′,5′-cyclic nucleotide phosphodiesterase (EC 3.1.4.1) (Boehringer Mannheim and Sigma Chemical Co.).

Synthesis of cAMP by a particle fraction from rat liver

Male Wistar rats (250–300 g body weight), fed a standard laboratory chow, were anaesthesized with ether and a liver sample excised and homogenized at 0–4°C with a teflon–glass Potter Elvehjem homogenizer in 9 volumes (ml/g sample weight) 0.25 *M* sucrose containing 10 m*M* Tris–HCl (pH 7.4) and 2 m*M* magnesium chloride. The homogenate was centrifuged at 0° and 43500 × *g* for 20 min, and the pellet suspended to the volume of the first homogenate in 20 m*M* glycylglycine–HCl (pH 7.5) containing 2 m*M* magnesium chloride and centrifuged (0°, 43500 × *g*, 20 min). The pellet obtained was the enzyme preparation used.

Portions of this preparation (about 4 mg protein as determined by the method of LOWRY[32], using bovine albumin as a standard) were incubated at 30° for 10 min in 400 μl 25 m*M* Tris–HCl (pH 7.5) containing 20 m*M* theophylline, 6 m*M* magnesium chloride, 4 mM ATP, [^{14}C]ATP (0.78 μCi/ml), 0.6 m*M* cAMP, [^{3}H]cAMP (to calculate recovery), without or with addition of either 10 m*M* sodium fluoride, $1.4 \cdot 10^{-2}$ m*M* glucagon or $5 \cdot 10^{-2}$ m*M* adrenaline. The incubation was terminated by heating the tubes in boiling water for 3 min. The tubes were centrifuged and the supernatants concentrated by evaporation and transferred to the chromatography paper strips.

Paper chromatography and enzymic conversion of cAMP to AMP

Descending chromatography was carried out on Whatman No. 1 paper (4 × 46 cm) with 96% ethanol–2 *M* ammonia–2 *M* acetic acid (100:19:21), pH 7.15. The chromatograms were developed for 18–22 hours at 19° (system 1). The spots were visualized by UV light, and the cAMP zone was cut into pieces and packed into a 1-ml disposable syringe to form a column of about 5 × 10 mm. This column was eluted by forcing 0.6–1 ml 10 m*M* glycylglycine–hydrochloric acid (pH 8.3) containing 2 m*M* magnesium chloride through the syringe. The pH in the eluate was adjusted to about 7.5 and 10 mU of beef heart 3′,5′-cyclic nucleotide phosphodiesterase were added. The mixture was incubated at 37° for 30 min, concentrated by evaporation and transferred to 4 × 46 cm Whatman No. 1 chromatography paper strips and developed as described above.

Liquid scintillation counting

The zones of the chromatograms were cut into pieces and transferred to counting vials, 0.5 ml 0.5 *N* H_2SO_4 or 0.5 ml 0.5 *N* HCl was added followed by 15 ml of the

scintillation fluid: BBOT–toluene–ethyleneglycolmonomethylether–naphthalene (4: 600:400:80, w/v/v/w)[33]. The vials were shaken vigorously for 5 min. The treatment with either acid gave a nearly quantitative elution of AMP from the paper. The presence of 0.5 ml of 0.5 *N* sulphuric acid (when glass vials were used) or 0.5 *N* hydrochloric acid (when polyethylene vials were used) counteracted the decrease of count rate otherwise observed with time[34]. The counting was performed in a Packard Tricarb Liquid Scintillator Spectrometer Model 3003.

RESULTS

The R_F values of purine derivatives in the chromatographic system described above (system 1) are listed in Table I. This separation system was tried for purifying the cAMP formed on incubation of liver particles with [^{14}C]ATP as described in the MATERIALS AND METHODS section. When these chromatograms were inspected under a UV lamp, the cAMP zone appeared to be completely separated from other UV absorbing materials (Fig. 1A). The radioactivity of this zone, however, overlapped that of the adjacent zones (Figs. 1B and C), and constituted less than 0.5 % of the total radioactivity of the chromatograms (in Figs. 1B and C the radioactivity is plotted using a logarithmic scale).

The adenosine region contained a large proportion of the total [^{14}C]radioactivity: 35–37 % when the assay was carried out in the absence of fluoride and 15 % when 10 m*M* sodium fluoride were present. This region was shown, by other chromatographic procedures[15], to contain more than one substance (adenosine and/or adenine and inosine and/or hypoxanthine). Re-chromatographing the cAMP fraction in the same system (system 1) revealed that the ^{14}C-labelled contamination from the

TABLE I

R_F VALUES OF PURINE DERIVATIVES CHROMATOGRAPHED ON WHATMAN NO. 1 PAPER

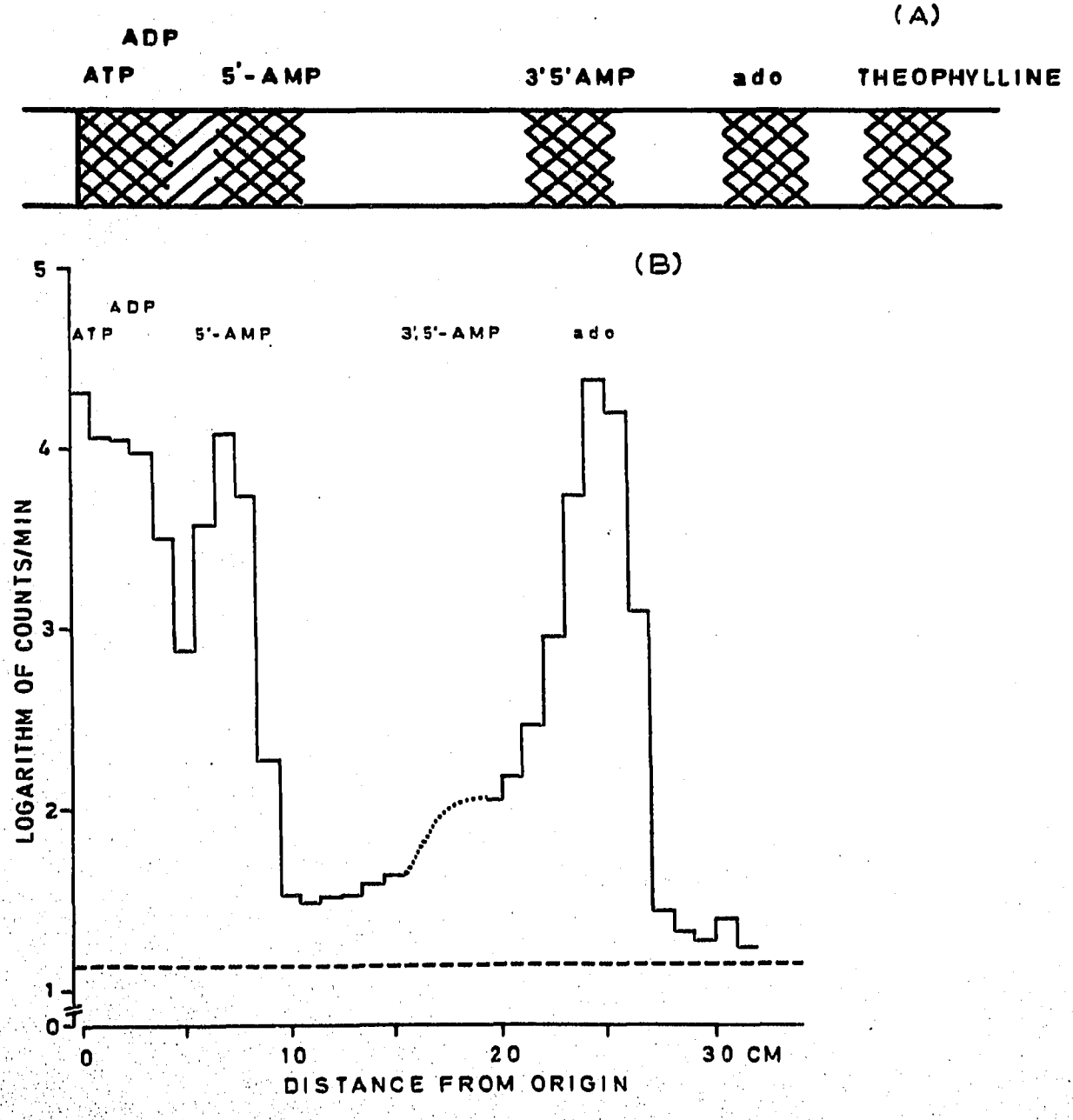
Solvent system: 96% ethanol–2 *M* ammonia–2 *M* acetic acid (100:19:20) pH 7.15.

Derivative	R_F
ATP	0.03
ADP	0.04
AMP	0.11
cAMP	0.33
2′,3′-AMP	0.36
Adenosine	0.51
Adenine	0.54
GTP	0.01
cGMP	0.27
Guanosine	0.44
IMP	0.10
cIMP	0.29
Inosine	0.49
Hypoxanthine	0.53
XMP	0.03
Xanthosine	0.28
Xanthine	0.39
Uric acid	0.07
Theophylline	0.69

adenosine region was about 30 % of the total ^{14}C-radioactivity when the incubation had been carried out in presence of 10 m*M* sodium fluoride, and more than 60 % in the absence of sodium fluoride (Fig. 2).

The re-chromatograms (Fig. 2) contained no radioactivity in the region of ATP, ADP and AMP. Therefore, in subsequent experiments, the eluate from the cAMP zone of the first chromatogram was incubated with cyclic nucleotide phosphodiesterase and rechromatographed in the same system (Fig. 3). The AMP specifically formed from cAMP thus migrated to a zone completely devoid of other labelled compounds. As can be seen from Fig. 3 the cAMP zone from the first chromatogram contained labelled material which was not converted to AMP on treatment with phosphodiesterase.

On the basis of these preliminary experiments the following method for de-



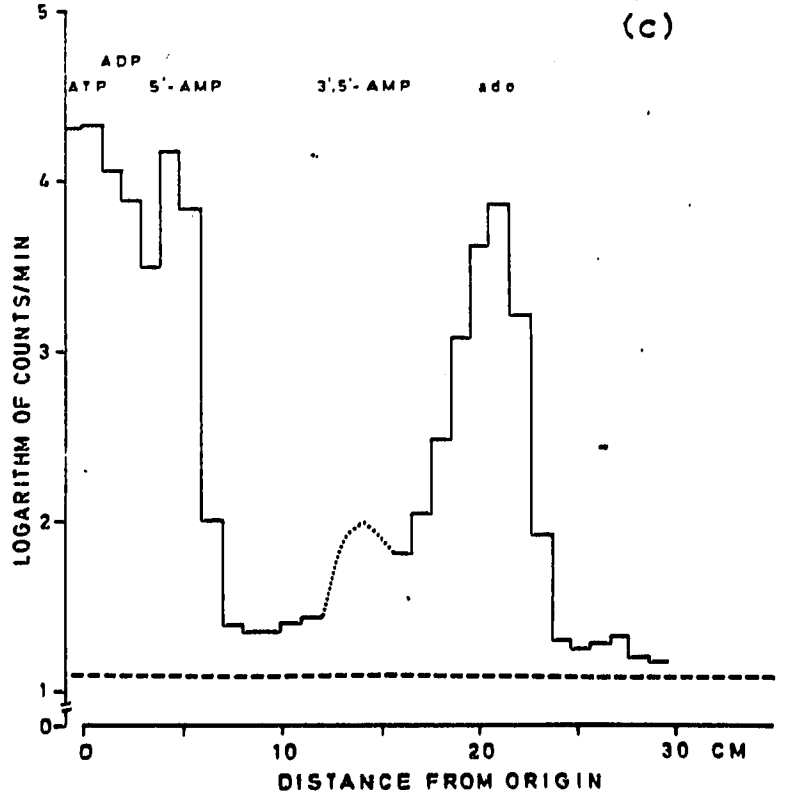


Fig. 1. Separation of metabolites from incubation mixtures of rat liver adenyl cyclase assays. The supernatants were chromatographed in system 1 (see text). (A) Schematic presentation of the chromatogram visualized by UV light. The different fractions are marked by appropriate abbreviations; ado = the adenosine region (adenosine, adenine, inosine, hypoxanthine). (B) (C) Presentation of the radioactivity (logarithmic plots) when 1-cm broad strips of the chromatogram were counted. The cAMP zone was removed for the further procedure. – – – –, Background count rate; ·····, calculated ^{14}C-radioactivity in the cAMP zone (based on data from Fig. 2). (B) Rat liver adenyl cyclase stimulated by glucagon ($1.4 \cdot 10^{-5}$ M). (C) Rat liver adenyl cyclase stimulated by NaF (10^{-2} *M*).

termination of labelled cAMP was adopted: (1) The supernatant from the incubation mixture or tissue extract is chromatographed on Whatman No. 1 paper with 96% ethanol–2 *M* ammonia–2 *M* acetic acid (100:19:21) pH 7.15, for at least 18–22 hours at 19°, in order to obtain optimal separation of cAMP from AMP, ADP and ATP (system 1); (2) The cAMP zone is eluted and treated with cyclic nucleotide phosphodiesterase in order to transform cAMP to AMP; (3) After treatment with phosphodiesterase the samples are rechromatographed in system 1; the labelled AMP formed from cAMP is thus specifically separated from metabolites which tend to co-chromatograph with cAMP; (4) The ^{14}C-activity and ^{3}H-activity of the final AMP fraction are counted and the amount of [^{14}C]cAMP calculated.

Because a known amount of [^{3}H]cAMP is included in each sample to correct for loss of cAMP, each step is performed in a semiquantitative way, giving a total recovery of about 30%.

The purity of the final AMP fraction was confirmed by descending chromatography on Whatman No. 1 paper with isobutyric acid–conc. ammonia–water (100:23:50) (system 2) and ascending chromatography on silica gel impregnated glass microfibre sheets (ITLC, type SG, Gelman Instrument Company) with *n*-heptane–acetone–isopropanol–0.03 *M* ammonium bicarbonate(50:50:200:47) (sys-

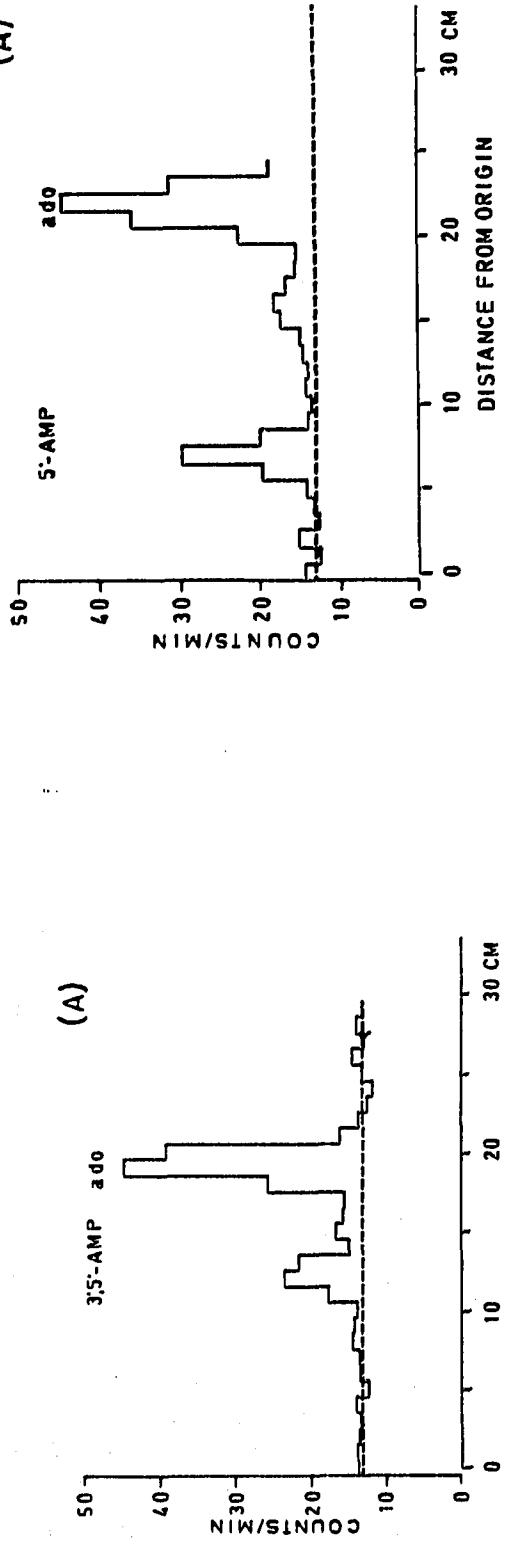


Fig. 2. Re-chromatography in system 1 of cAMP fractions from: (A) The chromatogram presented in Fig. 1B. (B) The chromatogram presented in Fig. 1C. – – –, Background count rate.

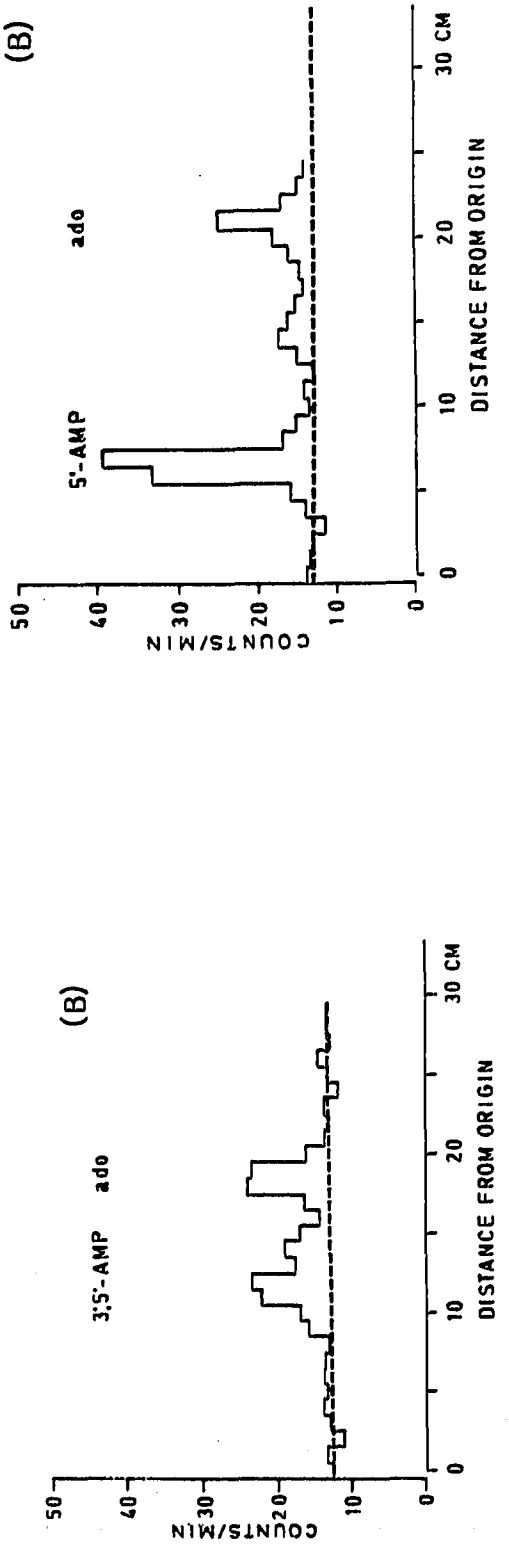


Fig. 3. Re-chromatography in system 1 of cAMP fractions after incubation with cyclic phosphodiesterase. (A) A cAMP fraction corresponding to that shown in Fig. 2A. (B) A cAMP fraction corresponding to that shown in Fig. 2B. – – –, Background count rate.

TABLE II

ADENYL CYCLASE ACTIVITY OF COARSE PARTICLE FRACTION FROM RAT LIVER

Adenyl cyclase activity was assayed at pH 7.5 in presence of 20 *mM* theophylline as described in the text. The results are mean ± one standard deviation. The number of observations are given in brackets. The reaction blank has been substracted.

Addition	*cAMP* (10^{-12} *moles/10 min*)	
	Per mg tissue weight	*Per mg protein*
None	3.0 ± 1.1	37 ± 13 (4)
Adrenaline $5 \cdot 10^{-5}$ *M*	3.9 ± 1.5	48 ± 18 (4)
Glucagon $1.4 \cdot 10^{-5}$ *M*	17.4 ± 0.8	214 ± 10 (3)
Sodium fluoride 10^{-2} *M*	22.3 ± 0.7	274 ± 8 (3)
Reaction blank[a]	0.4 ± 0.3	5 ± 4 (4)

[a] Incubation mixture heated in boiling water bath for 3 min before incubation.

tem 3). The specificity of the method has been further tested by performing the liver assay with [^{3}H]AMP added instead of [^{3}H]cAMP. No ^{3}H-radioactivity was found in the final AMP fraction, confirming that no AMP breakdown products present in the cAMP zone (*e.g.* xanthosine or xanthine) are converted to components migrating close to AMP (*e.g.* uric acid) during or after the treatment with phosphodiesterase.

Adenyl cyclase activity in the liver particle fraction described in the MATERIALS AND METHODS section, assayed by this method, is presented in Table II.

DISCUSSION

Paper chromatography similar to system 1 above has been used as the only separation procedure for isolation of cAMP from biological material[21–26]. Whether the cAMP fraction thus obtained is sufficiently pure probably depends on the experimental conditions. The pattern and amount of enzymes which act on adenine nucleotides and their metabolites differ in various tissues and tissue fractions. Further, the pattern of labelled metabolites formed from [^{14}C]ATP is quite different from that formed from [α-^{32}P]ATP. Finally, an active ATP-regenerating system may reduce the amount of breakdown products formed during incubation[25]. Therefore, a particular separation method may be adequate for one type of experiment, but quite inadequate under other conditions.

The activity of rat liver adenyl cyclase found in the present study is about one third of that obtained by SUTHERLAND *et al.*[1] in a homogenate from dog liver and similar to that obtained by BÄR AND HAHN in a particulate fraction from rat liver[35]. The small effect of adrenaline, compared to glucagon and F^-, has also been observed by others[30,37]. Other experiments in our laboratory indicate that the sensitivity of liver adenyl cyclase to adrenaline is easily lost on preparation of the particle fraction.

Adenyl cyclase activities of rat liver homogenates 10–15 times higher than those reported here were recently reported by HOMMES AND BEERE[38], who measured the amount of [^{14}C] after isolation of the cyclic nucleotide by the chromatographic method developed by RABINOWITZ *et al.*[12]. This is a one-step chromatographic sep-

aration on Whatman 3MM paper. RABINOWITZ *et al.* found this procedure to be adequate for separating cAMP from other labelled compounds formed from [α-^{32}P]ATP by a particle fraction from skeletal muscle. As shown by DOUŠA AND RYCHLÍK[3] this method does not separate cAMP from inosine and hypoxanthine. We have found that during incubation with liver enzyme preparation large amounts of inosine and/or hypoxanthine are formed. Therefore the discrepant results obtained by HOMMES AND BEERE[38] may at least in part be due to contamination of their cAMP fraction. Other examples of discrepant results due to inadequate separation have been reported by EMMELOT AND BOS[6] and by BÄR AND HECHTER[11].

The chromatographic system routinely used by us (system 1) does not completely separate cyclic 3',5'-inosine monophosphate and cyclic 3',5'-guanosine monophosphate from cAMP and IMP, and GMP from AMP. Both the cyclic compounds are metabolized by beef heart phosphodiesterase at the conditions used. Thus, if they are formed in substantial amounts during incubation, they might contribute to the radioactivity of the final fraction. However, we did not find support for any such production.

A combination of chromatography and incubation with phosphodiesterase has been used previously for checking the purity of cAMP fractions[5,18,26]. Enzymatic conversion of cyclic 3',5'-guanosine monophosphate (cGMP) to guanosine has been used in a procedure for determination of cGMP based on the different chromatographic behaviour of cGMP and guanosine[30,40].

The method described here has proved itself particularly useful for measuring labelled cAMP under conditions where many other labelled metabolites are formed. In addition to determination of adenyl cyclase activity in particulate preparations, the method has been used for measuring cAMP formed from endogenous ATP labelled by exposing skin pieces to [^{14}C]adenine (ref. 41) and cardiac muscle and liver slices to [^{14}C]adenine and [^{14}C]adenosine (to be published). The procedure is somewhat time-consuming, but relatively simple to perform. As in the case of other methods, its validity should be examined whenever applied to new and different experimental conditions.

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