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PRELIMINARY STUDY OF THE USE OF DANSYL CHLORIDE TO DETERMINE CYCLIC-3',5'-AMP IN TISSUES

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SUMMARY

A highly sensitive method for the determination of cyclic-3',5'-AMP has been developed which involves the reaction of the substance with dansyl chloride and the subsequent separation of the dansyl-cyclic-3',5'-AMP derivative by thin-layer chromatography. Experiments with standard solutions of ^{3}H-cyclic-3',5'-AMP have shown that there is a direct relationship between the amount of dansyl-^{3}H-cyclic-3',5'-AMP recovered and that dansylated. The procedure is exceedingly sensitive, allowing milligram quantities of material to be analysed for its endogeneous cyclic-3',5'-AMP content. With the use of ^{14}C-adenine as substrate, this method permits the separation of ^{14}C-cyclic-3',5'-AMP formed from the substrate and other ^{14}C-containing compounds, thus allowing the turn-over of cyclic-3',5'-AMP to be studied. The usefullness of the method is demonstrated by analysing the turn-over and endogenous content of cyclic-3',5'-AMP in rat nervous tissue.

INTRODUCTION

The formation of cyclic-3',5'-AMP from ATP by the enzyme adenyl cyclase and the subsequent action of cyclic-3',5'-AMP have been postulated as essential steps in some of the metabolic and functional changes produced by a variety of hormones and neurotransmitters[1,2]. Two general procedures have been widely used for the analysis of adenyl cyclase, both of which are based on the isolation of radioactive cyclic-3',5'-AMP from a radioactive precursor such as adenine or ATP[3–6]. In the method of Krishna *et al.*[4] and Shimizu *et al.*[3], the bulk of the cyclic-3',5'-AMP formed is separated from ATP by chromatography on Dowex cation-exchange resin or thin-layer plates and the purified sample is then treated with nascent barium sulphate, which removes most of the remaining ATP and other contaminating materials. In the other method[5,6], the radioactive cyclic-3',5'-AMP is separated from its precursor by chromatography over aluminium oxide. Insofar as measurement of endogenous tissue levels of cyclic-3',5'-AMP is concerned, a wide variety of methods exists[7], and that most widely used because of its simplicity and sensitivity is that originally described by Gilman[8], which is based on a competitive protein binding assay.

The technique described in this paper makes use of the reagent 1-dimethylaminonaphthalene-5-sulphonyl (dansyl) chloride to convert cyclic-3′,5′-AMP into a fluorescent dansyl derivative, which can then be separated from other products by thin-layer chromatography. In order to test the validity of this technique and its applicability to biological systems, determinations were made not only of the amount of cyclic-3′,5′-AMP, but also of the turnover of cyclic-3′,5′-AMP from [^{14}C]adenine in the central nervous system of the rat. The major advantages of the procedure are its simplicity and the fact that it enables the cyclic-3′,5′-AMP content and turnover in tissue to be studied easily in the same sample.

EXPERIMENTAL

Radioactive chemicals

[^{14}C]Dansyl chloride (Schwartz/Mann, Orangeburg, N.Y., U.S.A.) had a specific activity of 98 mCi/mmole, [^{14}C]adenine (Radiochemical Centre, Amersham, Great Britain) 281 mCi/mmole and [^{3}H]adenine-3′,5′-cyclic phosphate (Radiochemical Centre) 17–23 Ci/mmole.

Dansylation

As the aim of the procedure was to measure not only the endogenous cyclic-3′,5′-AMP content of tissue using dansyl chloride but also the incorporation of radioactivity from adenine into cyclic-3′,5′-AMP, it was necessary to examine cyclic-3′,5′-AMP together with other possible substances formed from adenine (see Table I). The actual procedure used for dansylation of the various substances was as described for amino acids[9,10]. Individual substances, previously solubilised in water, ethanol or 0.1 *N* hydrochloric acid, were subsequently adjusted to pH 9 with 0.5 *N* sodium hydrogen carbonate solution so that concentration ranges of between 1 and 100 μmole/ml were achieved. An aliquot of the sample was mixed with an equal volume of dansyl chloride in acetone (saturated at room temperature and sometimes mixed with [^{14}C]dansyl chloride) and incubated for 30 min at 37° in the dark (the usual reaction volume was 20–50 μl).

Biological samples were obtained from male rats, which were decapitated and their brains rapidly dissected and placed in liquid nitrogen. Cylinders of tissue were then removed from the frozen brains, which were thawed slightly, using a "punch-like" system, weighed immediately and homogenised at 0° in 0.2 *N* perchloric acid. After centrifugation at 1000 *g* at 0° for 30 min, the acid from the supernatants was extracted five times with equal volumes of diethyl ether. Each aqueous phase was freeze-dried, re-suspended in sodium hydrogen carbonate solution (pH 9) and dansylated as described above.

Chromatography and analysis

The products of dansylation were separated by one- or two-dimensional chromatography on silica gel G plates (Merck 60). Known amounts of cyclic-3′,5′-AMP or metabolites of the nucleotide (10^{-13}–10^{-6} mole) were applied to a silica gel G plate and developed with *n*-butanol saturated with 0.1 *N* hydrochloric acid. The solvent system used for development in the second dimension was chloroform–methanol–acetone (15:4:1 by vol.). The individual spots were localised by ultraviolet irradiation, outlined in pencil and eluted from the silica gel with methanol. The extraction was

repeated seven times and the combined eluates from a single substance were dried in counting vials with a stream of nitrogen. Scintillation fluid (4 g of PPO and 0.1 g of POPOP per litre of toluene) was added to the vials and the radioactivity counted in a Packard scintillation spectrometer.

Formation of cyclic-3',5'-AMP from [^{14}C]adenine

Brains were removed from the rats as described above and small pieces of the hemispheres quickly chopped. The slices were pre-labelled with [^{14}C]adenine (1.5 μCi of adenine per 0.5–1 g of tissue per 10 ml of physiological saline) according to the method of Shimizu *et al.*[3]. The incubation medium used was that described by McIlwain[11], which consisted of 120 m*M* sodium chloride, 3 m*M* potassium chloride, 1.2 m*M* potassium dihydrogen orthophosphate, 1.2 m*M* magnesium chloride, 0.75 m*M* calcium chloride, 25 m*M* sodium hydrogen carbonate and 10 m*M* glucose, and was continuously oxygenated. After being pre-labelled for 40 min, the slices were washed rapidly and aliquots incubated for an additional 10 min in physiological saline alone or with a test substance (see Fig. 5). Following incubation, the slices were homogenised at 0° in 0.2 *N* perchloric acid and, after centrifugation and subsequent removal of the acid, the products were dansylated as described above.

RESULTS AND DISCUSSION

Dansylation and chromatography

In comparison with the other nucleotides dansylated, cyclic-3',5'-AMP reacted readily and easily with dansyl chloride (Table I). Only small yields of dansylated ATP, ADP, AMP and cyclic-2',3'-AMP were recovered, even when the dansylation reaction time was increased to 24 h. It is not certain why cyclic-3',5'-AMP is dansylated so easily compared with the other nucleotides, although it may simply be due to the structure of the molecule. In any event, this interesting observation makes it possible to determine the cyclic-3',5'-AMP content of a sample merely by using dansyl chloride. Fig. 1 shows that the dansylation of cyclic-3',5'-AMP is a linear function of content. In these experiments, between 10^{-13} and 10^{-9} mole of a solution of [^{3}H]cyclic-3',5'-AMP was mixed with a known amount (10^{-4} mole) of cold cyclic-3',5'-AMP and each sample subjected to reaction with an excess of dansyl chloride. Thereafter the whole of the contents were either placed in a counting vial (for construction of a theoretical curve) or applied quantitatively to a silica gel plate and, after chromatography, the cyclic-3',5'-AMP spot was removed and analyzed by scintillation spectrometry (experimental curve) as described under Experimental.

When using silica gel G plates, two developing solvent systems were required in order to separate cyclic-3',5'-AMP from other possible substances that were also metabolized from adenine by nervous tissue (see Table I). With these two systems, it was also possible to separate the major amino acids normally present in the brain from cyclic-3',5'-AMP (see Fig. 2). When fractionating dansyl-cyclic-3',5'-AMP on 5 × 5 cm silica plates, as little as 10^{-9} mole could be easily detected under UV light. It could also be separated from most of the other substances by one-dimensional chromatography on silica gel plates (Fig. 3), although dansyl-cyclic-2',3'-AMP could not. However, as cyclic-2',3'-AMP is very poorly dansylated, one-dimensional chro-

TABLE I

RECOVERY (%) COMPARED WITH THE THEORETICAL VALUE (100%) WHEN REACTING 10^{-3} OR 10^{-5} *M* OF SUBSTANCE WITH EXCESS OF [^{14}C]DANSYL CHLORIDE

In these experiments 10^{-6} or 10^{-8} mole/ml of substance in sodium hydrogen carbonate solution (0.5 *N*, pH 9) was made to react with excess of [^{14}C]dansyl chloride for 30 min at 37° (see Experimental). A defined aliquot was chromatographed, the dansylated product eluted and the radioactivity measured and compared with the theoretical value when assuming complete dansylation and recovery. Each value given is the mean from three separate experiments.

Substance	*Recovery (%)*	
	10^{-3} M	*10^{-5} M*
Adenine	86	74
Adenosine	83	66
Cyclic-3′,5′-AMP	69	58
Cyclic-2′,5′-AMP	20	20
2′-AMP	20	20
3′-AMP	20	20
AMP	20	20
ADP	20	20
ATP	44	20
Hypoxanthine	75	72
Inosine	87	80
IMP	20	20
Xanthosine	84	86
Guanine	79	72
Guanosine	65	61
Cytidine	60	71
Cytosine	44	48

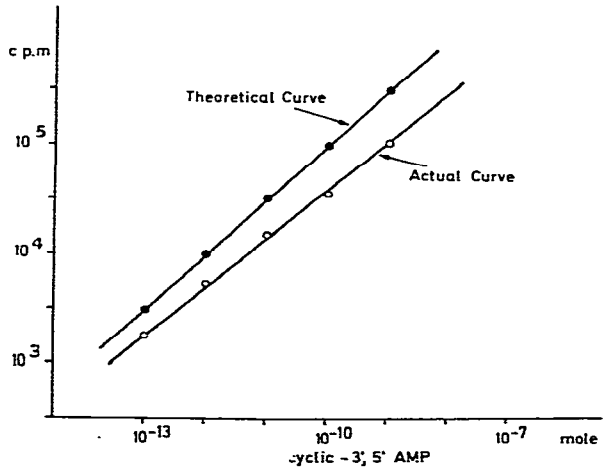


Fig. 1. Calibration graph for the determination of cyclic-3′,5′-AMP in the range 10^{-13}–10^{-9} mole. Each point is the average of 7 separate analyses. It can be seen that the actual curve differs from the theoretical or expected curve, and this has to be taken into consideration in the final calculations.

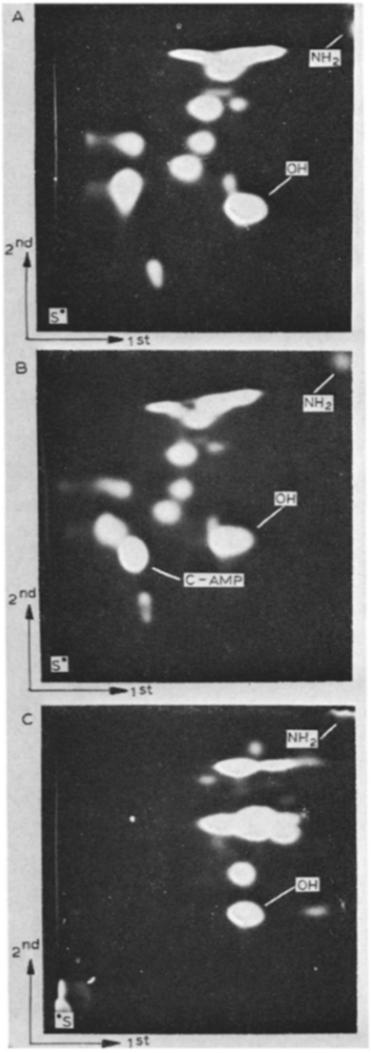


Fig. 2. Two-dimensional chromatographic separation of dansyl-cyclic-3′,5′-AMP from possible metabolites and a mixture of dansylated amino acids (lysine, histidine, arginine, aspartate, threonine, serine, glutamate, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine) on silica gel thin-layer plates (5 × 5 cm). The developing solvent in the first dimension was *n*-butanol saturated with 0.1 *N* HCl and in the second dimension chloroform–methanol–acetic acid (15:4:1 by vol.). S = starting point; OH = dansyl-OH; NH_2 = dansyl-NH_2; C-AMP = dansyl-cyclic-3′,5′-AMP. Chromatogram A shows the separation of the mixture of dansyl-amino acids, while chromatogram B shows the clear separation of dansyl-cyclic-3′,5′-AMP from this mixture of dansyl-amino acids. Chromatogram C shows the fractionation of a mixture of dansylated metabolites of cyclic-3′,5′-AMP (adenosine, adenine, cyclic-2′,3′-AMP, 2′-AMP, 3′-AMP, AMP, ADP, ATP, hypoxanthine, inosine, IMP, xanthosine, guanine, guanosine, cytidine and cytosine), where it can be seen that none of these substances would co-chromatograph with dansyl-cyclic-3′,5′-AMP (compare with chromatogram B).

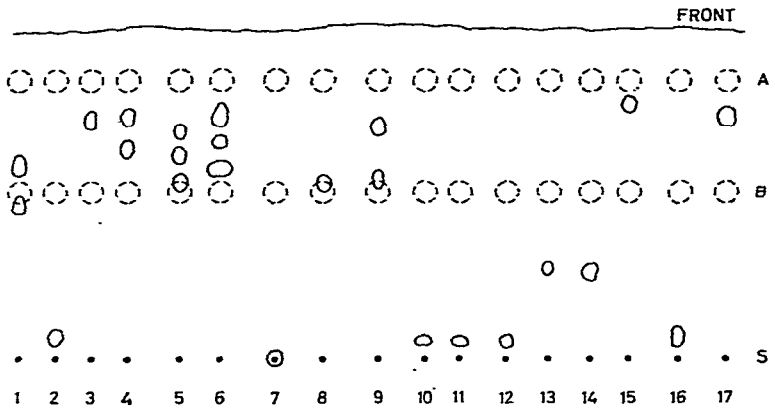
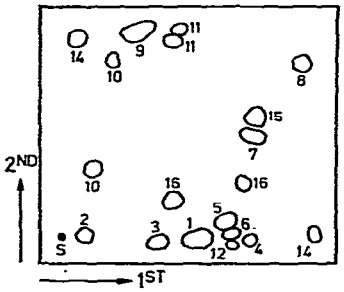


Fig. 3. One-dimensional chromatographic separation of dansyl-cyclic-3′,5′-AMP and metabolites on a silica gel thin-layer plate (15 × 15 cm). The solvent system used was *n*-butanol saturated with 0.1 *N* HCl. A = dansyl-NH_2; B = dansyl-OH. S = starting point; 1 = dansyl-xanthosine; 2 = dansyl-ADP; 3 = dansyl-guanine; 4 = dansyl-guanosine; 5 = dansyl-adenosine; 6 = dansyl-inosine; 7 = dansyl-ATP; 8 = dansyl-cytidine; 9 = dansyl-hypoxanthine; 10 = dansyl-2′-AMP; 11 = dansyl-3′-AMP; 12 = dansyl-IMP; 13 = dansyl-cyclic-2′,3′-AMP; 14 = dansyl-3′,5′-AMP; 15 = dansyl-cytosine; 16 = dansyl-AMP; 17 = dansyl-adenine. Note: more than one dansyl derivative is formed for compounds 1, 4, 5, 6 and 9.

matography can be a useful method for the initial purification, especially when many samples have to be analyzed.

If polyamide plates (F 1700 TLC-ready plastic sheets from Schleicher & Schüll, Dassel, G.F.R.) are used to chromatograph dansyl-cyclic-3′,5′-AMP, the amount of substance detectable under UV light can be decreased by a factor of about



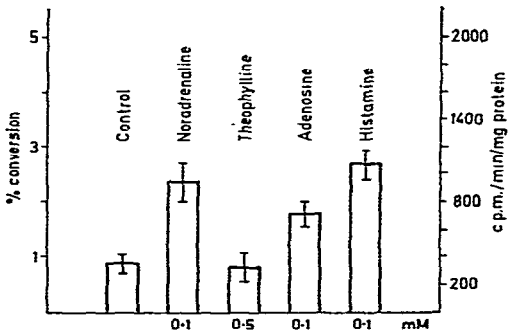


Fig. 4. Two-dimensional chromatographic separation of dansyl-cyclic-3′,5′-AMP and some metabolites on a polyamide layer (3 × 3 cm). The solvent systems used were water–formic acid (100:3, v/v) in the first dimension and benzene–acetic acid (9:1, v/v) in the second dimension. S = starting point; 1 = dansyl-OH; 2 = dansyl-ATP; 3 = dansyl-ADP; 4 = dansyl-AMP; 5 = dansyl-cyclic-3′,5′-AMP; 6 = dansyl-cyclic-2′,3′-AMP; 7 = dansyl-NH_2; 8 = dansyl-adenosine; 9 = dansyl-adenine; 10 = dansyl-guanine; 11 = dansyl-hypoxanthine; 12 = dansyl-IMP, -3′-AMP, -2′-AMP; 14 = dansyl-cytidine; 15 = dansyl-cytosine; 16 = dansyl-xanthosine.

Fig. 5. Incorporation of radioactivity from [^{14}C]adenine into rat cortical cyclic-3′,5′-AMP (control) and the effect of various substances on the degree of incorporation. Slices of rat cortex were pulse labelled with [^{14}C]adenine (40 min) and then incubated with (10^{-4} *M*) or without test substances for 10 min (see Experimental). Thereafter the tissue cyclic-3′,5′-AMP was assayed by dansylation, the standard error of the mean being calculated for six experiments.

100. With the two developing solvent systems normally used for dansyl-amino acids, it can be seen from Fig. 4 that dansyl-cyclic-3',5'-AMP is not clearly fractionated from other possible metabolites that could be metabolised from adenine (see Table I), and that it runs in the same position as dansyl-aspartate[9,10]. It is thus clear that other developing solvents will have to be found if polyamide layers are to be employed for the clear separation of dansyl-cyclic-3',5'-AMP from most of the dansyl-amino acids and other metabolites as achieved for the silica gel plates.

Use of the method to determine the cyclic-3',5'-AMP content of tissue

[^{14}C]Dansyl chloride was used to measure the endogenous amount of cyclic-3',5'-AMP in tissue. Great care was taken to dissect the tissues as quickly as possible below 0° before homogenization in perchloric acid, as it is known that rapid *post mortem* changes take place with cyclic-3',5'-AMP[12]. The normal procedure was to produce a series of tubes, each containing a definite amount of homogenate and to which were added varying amounts of internal standard cyclic-3',5'-AMP. Following extraction and reaction of the contents of each tube with [^{14}C]dansyl chloride as described under Experimental, about 2 μl of dansylated substances (corresponding to approximately 10 mg of tissue) were applied to a silica gel plate. After two-dimensional chromatography, the spot corresponding to dansyl-cyclic-3',5'-AMP was analyzed by scintillation spectrometry and the content of cyclic-3',5'-AMP was calculated from the known specific activity of the [^{14}C]dansyl chloride added. The recovery of the procedure, which had been previously determined by adding [^{3}H]-cyclic-3',5'-AMP to tissue samples and dansylating with cold dansyl chloride, was also used in the calculations; it was found to be 47 $\pm$ 5% (standard error of the mean for 16 experiments). By using this procedure, the content of cyclic-3',5'-AMP determined in the cerebellum, mesencephalon and cortex of the rat (Table II) was found to be of the same order as that described by other workers[13,14].

TABLE II

CYCLIC-3',5'-AMP CONTENT (nmole/g) OF VARIOUS BRAIN REGIONS

Values (corrected for recovery) are means $\pm$ S.E.M. for 8–10 experiments.

Brain region	*Extract directly dansylated*	*Extract initially purified by the procedure described by Kneer et al.[15] before dansylation*
Cerebellum	2.172 $\pm$ 0.721	1.864 $\pm$ 0.711
Mesencephalon	1.754 $\pm$ 0.639	1.521 $\pm$ 0.629
Cortex	1.021 $\pm$ 0.541	0.861 $\pm$ 0.413
Whole brain	1.631 $\pm$ 0.551	1.496 $\pm$ 0.501

In order to see whether the tissue cyclic-3',5'-AMP level was too high, owing to possible contamination by unknown tissue substances that are also dansylated and that may have the same chromatographic mobility as dansyl-cyclic-3',5'-AMP, the following experiments were additionally carried out. Firstly, the spot corresponding to dansyl-cyclic-3',5'-AMP on the chromatograms was re-chromatographed using one or a combination of the solvent systems ethyl acetate–methanol–acetic acid (20:1:1 by vol.), diethyl ether–methanol–acetic acid (100:5:1 bij vol.) or *n*-butanol saturated with ammonia. In each instance only a single spot corresponding to dansyl-

cyclic-3',5'-AMP was observed. Secondly, samples prior to dansylation were passed through columns containing Bio-Rad AG 1-X8 formate (200–400 mesh) and the cyclic-3',5'-AMP was eluted with formic acid as described by Kneer *et al.*[15]. In both instances the content of cyclic-3',5'-AMP was found to be slightly lower than that determined by straightforward chromatography of the dansyl derivatives (see Table II). However, the differences in the values were less than 15% and, because of the small amounts of cyclic-3',5'-AMP present in the tissues, it seems that the additional steps may be omitted.

Use of [^{14}C]adenine as a pulse labelling precursor

In these experiments the tissue cyclic-3',5'-AMP was pre-labelled by incubating the tissue with [^{14}C]adenine and then determining the influence of substances on the incorporated radioactivity. After extracting the cyclic-3',5'-AMP and other substances from the tissue as described under Experimental, the contents were subjected to reaction with cold dansyl chloride. The incorporation of radioactivity from [^{14}C]adenine into [^{14}C]cyclic-3',5'-AMP was easily determined because other possible metabolites of [^{14}C]adenine (see Table I) which contain some radioactivity are clearly fractionated as dansyl derivatives from dansyl-cyclic-3',5'-AMP by two-dimensional chromatography on silica gel layers (see Fig. 2).

Fig. 5 shows that less than 1% of the radioactivity from [^{14}C]adenine is incorporated into rat cortical cyclic-3',5'-AMP under the conditions used. This extent of incorporation is comparable to the values generally described for other methods[3,16]. Moreover, it can be seen from Fig. 5 that noradrenaline, histamine and adenosine stimulated the formation of cyclic-3',5'-AMP, while theophylline, at the concentration used, had little effect. These results are in general agreement with published data[2].

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