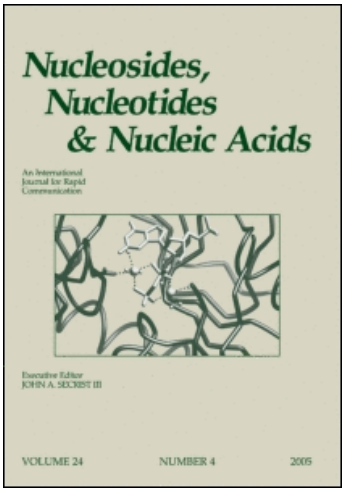

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SYNTHESIS OF 5'-O-SUCCINYL-2',3'-CYCLIC ADENOSINE MONOPHOSPHATE

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ABSTRACT: 5'-O-Succinyl-2',3'-cyclic adenosine monophosphate was synthesized in preparation for 2',3'-cyclic nucleotide antibody production. Succinylation was performed with [^{14}C]labeled and unlabeled succinic anhydride and morpholinodicyclohexylcarbodiimide. The products, resolved on QAE Sephadex, were purified and characterized by comparison with authentic 3',5'-cyclic AMP succinyl derivatives. Yield: 73% monosuccinyl-(5'-O)-2',3'-cyclic AMP; 10% disuccinyl-(N^6,5'-O)-2',3'-cyclic AMP. The phosphodiester ring in the monosuccinyl derivative was shown to be intact by its susceptibility to alkaline phosphatase only after exposure to 2',3'- cyclic nucleotide 3'-phosphodiesterase.

The 2',3'-cyclic nucleotides are considered to be nonfunctional metabolites of RNA. Reports, however, of (i) high concentrations of 2',3'-cyclic GMP in bacteria[1], (ii) 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) activity in membranes[2,3], (iii) CNP participation in membrane synthesis[4], and (iv) 2',3'-cyclic phosphate participation in eucaryotic and viral RNA processing[5,6] suggest that the 2',3'-cyclic nucleotides warrant more detailed investigation.

Studies on the 2',3'-cyclic nucleotides have been hampered by the unavailability of derivatives for in vivo studies and the lack of sensitive methods for their measurement[7,8]. Thus, succinylated 2',3'-cyclic AMP was prepared for conjugation to protein for subsequent antibody production and to increase the lipophilicity of a 2',3'-cyclic nucleotide to probe its effects in situ.

RESULTS AND DISCUSSION

Several nucleotides have been acylated in preparation for immunization[9-12]. Adenine 2',3'-cyclic nucleotide was selected for succinyla-

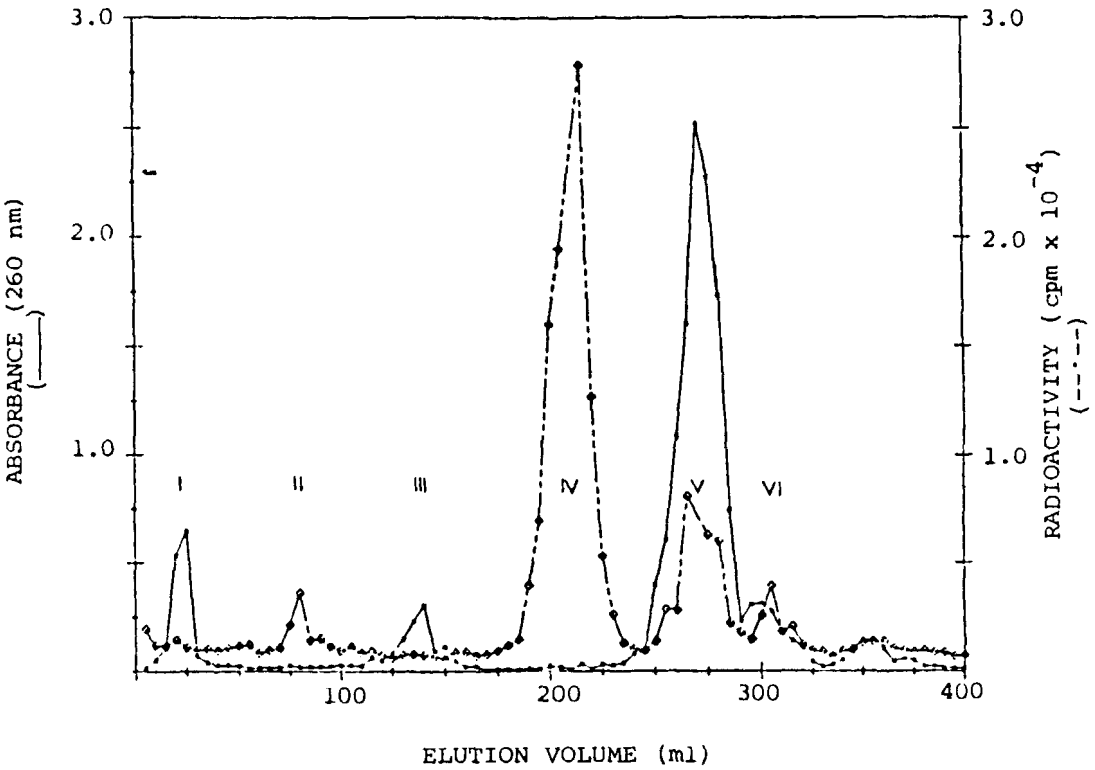

FIGURE 1. QAE SEPHADEX ELUTION OF SUCCINYLATION PRODUCTS
(I) pyridine/dioxane, (II) succinic anhydride, (III) 2',3'-cyclic AMP, (IV) succinic acid, (V) 5'-O-succinyl-2',3'-cAMP, (VI) N^6-5'-O-disuccinyl-2',3'-cAMP.

tion because of the (i) preference of CNP for adenine nucleotides, (ii) the prevalence of the adenine nucleotides in metabolism, and (iii) the importance of their well known 3',5'-cyclic phosphate isomers. Succinylation was performed according to the method described for 2'-acylation of 3',5'-cyclic nucleotides[9] modified to favor formation of 5'-O-succinyl-2',3'-cyclic AMP. Trace quantities were synthesized in the presence of [^{14}C]-succinic anhydride to facilitate identification and yield determination.

IDENTIFICATION: The succinylation products were chromatographed on QAE Sephadex. Six peaks absorbing UV light at 260nm and/or containing radioactivity were resolved (FIG 1). Peak I (15-30 ml) contained residual pyridine and dioxane. Peaks II (70-85 ml) and IV (185-240 ml) contained succinate (radioactivity) but no nucleotide (A_{260} absorbance).

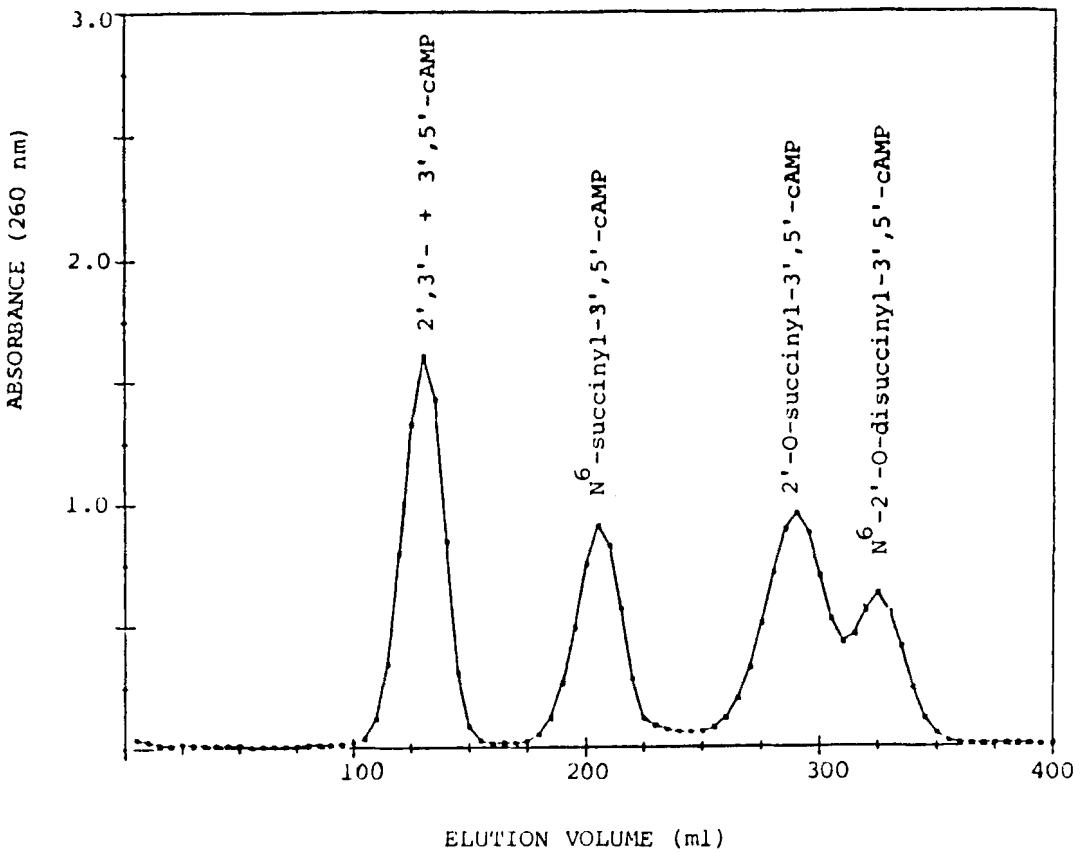

FIGURE 2. QAE SEPHADEX ELUTION OF NUCLEOTIDE STANDARDS
Flow rate 90 ml/hr; 5 ml fractions. Eluted with NaCl gradient (0.02-0.4M) in phosphate buffer (pH 7.0; 0.01M).

The latter were shown to be succinic anhydride and free succinic acid, respectively, by comparison with unhydrolyzed and hydrolyzed succinic anhydride (data not shown). Peak III (175-190 ml) contained A_{260} absorbing material but no radioactivity. This fraction was unreacted 2',3'-cyclic AMP as shown by comparison with authentic 2',3'-cyclic AMP and 3',5'-cyclic AMP (FIG 2). Peaks V (250-290 ml) and VI (300-325 ml) contained both nucleotide (A_{260}) and succinate (^{14}C). The succinate: nucleotide ratios, estimated from the extinction coefficient of 2',3'-cyclic AMP (ε_{mM} = 13.1) and specific radioactivity of succinic anhydride (12.3mC/mm), were 1:1 and 2:1, respectively. The nucleotide ratios, ionic charge on the succinylated derivatives at pH 7.0, the preferential

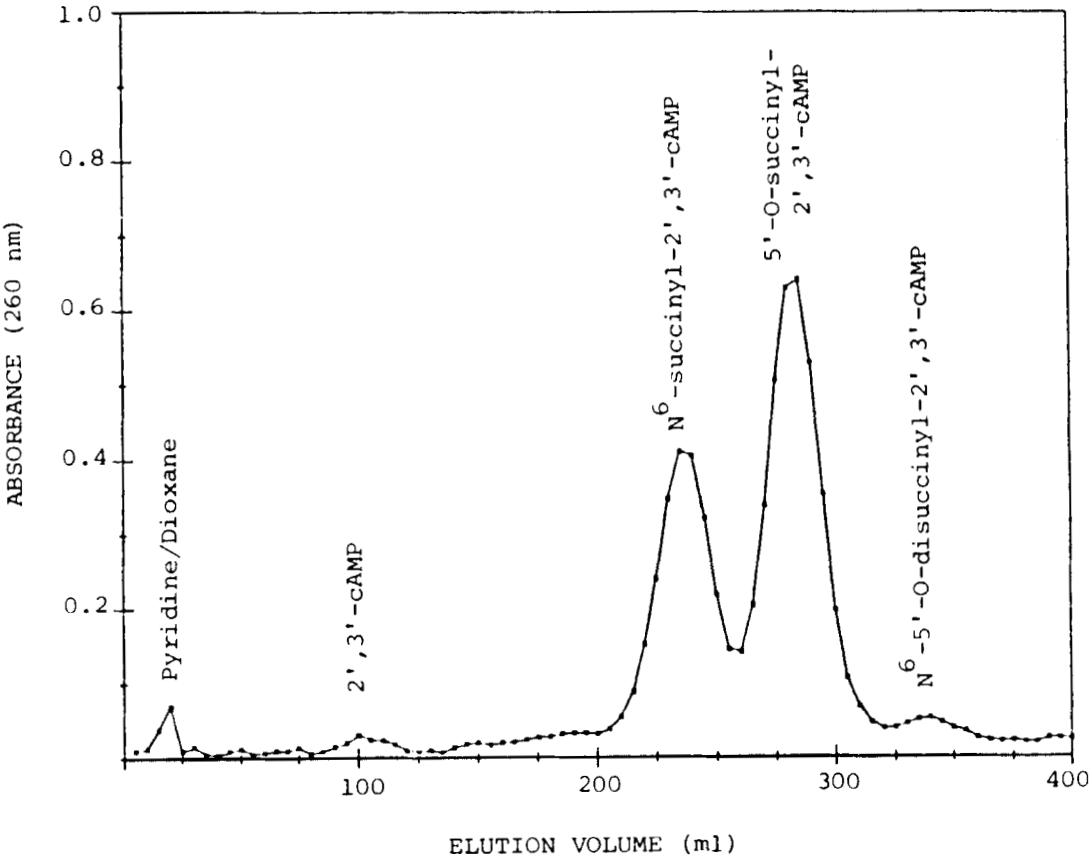

FIGURE 3. QAE SEPHADEX ELUTION OF SUCCINYLATION REACTION MIXTURE AFTER HYDROLYSIS FOR 72 HRS AT ROOM TEMP (FIG 1 conditions)

hydroxylation of the 5'-OH group, and the similar elution profile of authentic 3',5'-cAMP indicate that Peaks V and VI were mono- and disuccinylated, respectively.

To determine if N^6-monosuccinyl-2',3'-cyclic AMP was formed by hydrolysis of the disuccinylated product[10], the lyophilized reaction mixture was hydrolyzed (distilled H_2O, room temp; 72 hr) and chromatographed. This resulted in (i) decreased disuccinyl-2',3'-cyclic AMP (Peak VI, FIG 1), (ii) increased N^6-monosuccinyl-2', 3'-cyclic AMP, but (iii) no change in the 5'-monosuccinyl-2',3'-cyclic AMP (FIG 3). These data confirm that the major product was 5'-O-monosuccinyl-2',3'-cyclic succinyl-2',3'-cyclic AMP with little or no formation of N^6-monosuccinyl- or N^6-5'-O-disuccinyl-2'-3'-cyclic AMP.

TABLE 1. PRODUCT YIELD
Succinylated 2',3'-cyclic AMP was chromatographed on QAE Sephadex. Peak fractions were pooled and analyzed for radioactivity and absorbance at 260 nm. Percent yield was calculated by comparison with the total amount of material applied to the column.

PEAK # (FIG 1)	PRODUCT	% YIELD CPM	% YIELD ABSORBANCE
I	Pyridine/Dioxane	-	-
II	Succinic Anhydride	6.0	-
III	2',3'-cAMP	-	7.0
IV	Succinic Acid (free)	49.9	-
V	5'-O-Succinyl-2',3'-cAMP	17.0	72.9
VI	N^6-5'-O-Disuccinyl-2',3'-cAMP	5.7	10.1
	(TOTAL RECOVERED)	78.6	90.0

YIELD: Of the material applied to the column, 80% and 90% of the radioactivity and A_{260} absorbing materials were recovered, respectively (TABLE 1). Approximately 60% of the radioactivity appeared as free succinate and less than 10% as unhydrolyzed anhydride. Twenty percent of the label was in the 5'-O-monosuccinyl-2',3'-cyclic AMP with less than 10% appearing in N^6-5'-O-disuccinyl-2',3'-cyclic AMP. Thus, approximately 73% of the nucleotide was monosuccinylated in the 5'-OH position; less than 10% was disuccinylated.

CHARACTERIZATION: Pooled Peak V (FIG 1) fractions were desalted and incubated with purified CNP for 30 min at 37°C. The products were resolved by thin layer chromatography. Treatment of authentic 2',3'-cAMP and succinylated nucleotide resulted in two spots with R_f values corresponding to 2'-AMP and 2',3'-cAMP. Thus, the cyclic phosphodiester bonds either remained intact or were reformed during succinylation.

The mechanism of 3',5'-cyclic nucleotide acylation by succinic anhydride has been described by Falbriard *et al*[13]. The ribose 5'-hydroxyl forms an ester with the succinate whereas attack at the N^6 primary amine forms an amide linkage. The yield of each product depends upon the rate of acylation at each of these functional groups. Cailla *et al*.[9,14] reported that 2'-O esterification requires one hr incubation while formation of the amide derivative occurs within 10 hr. The rela-

tive amounts of ester and amide-linked products are also dependent upon the nucleophilicity of the solvents used during acylation. Exclusive use of pyridine tends to result in production of N-substituted nucleotides while dioxane, a non-nucleophilic solvent, tends to result in production of O-substituted products. In the present study, synthesis of 5'-O-succinyl-2',3'-cAMP was favored by using short (2 hr) incubation periods and by including dioxane as the solvent. These conditions were chosen to generate a product with minimal steric hinderance in the vicinity of the 2',3'-cyclic phosphodiester bonds and the purine ring.

The identity of the major succinylation product was determined by (i) charge considerations, (ii) co-chromatography of 3',5'-cyclic AMP standards, (iii) determination of succinate:nucleotide ratios, (iv) susceptibility to enzyme hydrolysis, and (v) UV, IR and NMR data.

EXPERIMENTAL

Succinylation: 2',3'-Cyclic AMP (57 μm; free acid; Boehringer Mannheim Biochemicals, Indianapolis, IN) was dissolved in 0.6 ml 4-morpholino-N,N'-dicyclohexyl-carbodiimide (57 μm; morpholine DCC; Sigma Chemical Company, St. Louis, MO) previously dissolved in hot pyridine. Radioactive 1,4[^{14}C]-succinic anhydride (50 μC; 4 nmol; 2.3 mC/mm; ICN, Irvine, CA), dissolved in 0.2 ml dioxane, was added and the mixture was incubated at room temperature for two hr. Unlabeled succinic anhydride (0.25 mmol in 0.2 ml dioxane) was added and incubation was continued for two hr. Unreacted succinic anhydride and mixed anhydrides were hydrolyzed with distilled water (10 ml). After lyophilization, the residue was dissolved in 4 ml of distilled H_2O. Preparative scale succinylation required 0.57 mmol 2',3'-cyclic AMP and morpholine DCC in 6 ml pyridine and 1.5 mmol succinic anhydride in 3 ml dioxane.

Characterization: The products of succinylation were resolved on QAE columns (1.5 x 15 cm) equilibrated with phosphate buffer (0.1 M, pH 7.0) containing 0.02 M NaCl. They were eluted with a phosphate buffered NaCl gradient (0.02-0.4 M; 4°C, 60-90 ml/hr). Five ml fractions were collected and monitored for absorbance (A_{260}) and radioactivity (Scinti-Verse II, Fisher Scientific Co., Santa Clara, CA). Authentic standards of 2',3'-cAMP, 3',5'-cAMP, N^6-succinyl-3',5'-cAMP, 2'-O-succinyl-3',5'-cAMP, and N^6-2'-O-disuccinyl-cAMP (Sigma Chemical Co.) were chromatographed under similar conditions. Fractions co-chromatographing with

the 2'-O-succinyl-3',5'-cAMP standard were lyophilized, desalted on Dowex 50-W8-400 and relyophilized. $(UV)_{max}$, H_2O (λ 259.5 nm), λ 260/λ280 = 5.92; IR (KBr) ν 3125-3550 (NH_2, OH), 2900-2950 (C-H), 1740 (COOH), 1660 (COOR), 1620, 1245 (P=O), 1190 (P-O), 1135 (P-O-C), 1085 (P-O-C), 925 (C-O-C); NMR (80mHz,D_2O) ^1H: 6.95 (m, 1, purine 3 or 8H), 6.83 (m, purine 3 or 8H), 4.83 (d, 2, 5'H, J = 5 Hz), 4.2-3.6 (m, 2, 2'H and 3'H), 3.46 (d, 1, 1'H, J = 3 Hz), 2.85 (d, 1, 4'H, J = 5 Hz), 2.22 (broad s, 4 succinyl Hs); NMR (36 mHz) ^{31}P: 21.478 ($\emptyset_3P$ = -6.0 ppm).

The integrity of the 2',3'-phosphodiester ring was assessed by its susceptibility to 2',3'-cyclic nucleotide phosphodiesterase (CNP) but not alkaline phosphatase. 5'-O-Succinyl-2',3'-cAMP was exposed to CNP (5 units at 37°C; 30 min) and/or alkaline phosphatase (3 units at 37°C; 30 min). The mixtures were chromatographed on silica gel[11] and developed with ethanol:(0.5 M) ammonium sulfate (5:2, v/v) before and after enzyme treatment. Product mobilities were compared with similarly treated standards of non-succinylated 2',3'-cAMP and 2'-AMP. The purified enzymes (Sigma Chemical Company) and were assayed against standard solutions of 2',3'-cyclic AMP and 2'-AMP prior to use.

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