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Instability of Succinyl Ester Linkages in $O^{2'}$-Monosuccinyl Cyclic AMP-Protein Conjugates at Neutral pH

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Chromatographic and immunological evidence is presented regarding the hydrolysis of the ester linkage of O^2-monosuccinyl cyclic AMP in neutral solutions Such hydrolysis occurs whether the nucleotide derivative is present in free form in solution or conjugated through its succinyl carboxyl group via an amide bond to proteins The latter process apparently occurs when succinyl cyclic AMP is conjugated to human serum albumin for use as an immunogen in the production of anti-cyclic AMP antibodies and when the derivative is coupled to the enzyme glucose-6-phosphate dehydrogenase (E C 1 1 1 49) The enzyme conjugate has been used in developing a homogeneous enzyme immunoassay for cyclic AMP Inhibition of the catalytic activity of enzyme-cyclic AMP conjugates by anti-cyclic AMP antibody decreases with time, apparently due to the loss of cyclic AMP from enzyme-cyclic AMP conjugates stored in neutral solutions In addition, the ability of free cyclic AMP to completely reverse the inhibition process decreases with time because of the presence of antibodies in the anti-cyclic AMP sera that apparently inhibit enzyme activity because of their binding specificity for the residual succinate-protein determinant sites of the enzyme conjugates Lyophilization of the conjugates immediately after preparation helps to overcome the problem, however, in vivo hydrolysis of immunogens prepared with the succinyl cyclic AMP derivative may always occur The consequence of this hydrolysis reaction and the subsequent formation of anti-succinyl-protein antibodies will be discussed with regard to existing RIAs for cyclic AMP and a new homogeneous enzyme immunoassay for the nucleotide

Key words *protein conjugates – ester linkage*

Introduction

The $O^{2'}$-monosuccinyl derivatives of nucleosides and nucleotides, particularly $O^{2'}$-monosuccinyladenosine 3′,5′-cyclic monophosphoric acid (SCAMP), have been employed to conjugate such molecules to carrier proteins for use as immunogens in the preparation of anti-nucleoside and anti-nucleotide antisera (Steiner et al, 1969, 1972), to enzymes for use in nucleotide heterogeneous enzyme immunoassays (EIA)

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(Yamamoto and Tsuji, 1980, Joseph and Guesdon, 1982) and to radiolabels for use as tracers in radioimmunoassays (RIA) of nucleotides (Steiner et al., 1969, 1972) In addition, a host of succinyl derivatives of steroids (Erlanger et al., 1957, 1959), anti-cancer agents (Okabayashi and Moffatt, 1982) and other drugs (Kawashima et al., 1976, Dixon, 1982) have been prepared for similar conjugation purposes

The instability of the nucleotide derivatives at high pH (pH 13) has been reported previously (Steiner et al., 1969, 1972) However, most immunoassay methodologies involving these derivatives are performed at or near neutral pH Further, upon injection into animals, immunogens prepared with the succinyl derivatives are also subjected to an in vivo neutral pH environment Despite the fact that RIAs for cyclic AMP and cyclic GMP have been in use for more than 15 years, no reports have appeared concerning the hydrolysis of the $O^{2'}$-ester linkages under these physiological conditions, nor the possible consequences of such hydrolysis It was, therefore, surprising to us that during our efforts to develop a homogeneous enzyme immunoassay for cyclic AMP, we found that free SCAMP as well as cyclic AMP-protein conjugates prepared with this derivative were unstable in neutral solution when stored at 4°C or even −20°C This report will provide evidence that this hydrolysis reaction occurs in protein-cyclic AMP conjugates used as immunogens in the preparation of anti-cyclic AMP antibodies and in enzyme-cyclic AMP conjugates that are potentially useful in homogeneous EIAs of cyclic AMP in biological fluids

Hydrolysis of the O^2-ester linkage of SCAMP in protein-SCAMP conjugates (prepared by carbodiimide (Steiner et al., 1969, 1972) or NHS-carbodiimide reaction procedures (Anderson et al., 1964)) (Fig 1) results in loss of the nucleotide to the solution, while the succinyl group remains bound to the protein, presumably through an amide bond to a lysyl residue of the protein There are at least 2 possible consequences when such a reaction occurs in cyclic AMP-protein immunogens during storage in solution prior to immunization or in vivo during slow release of the conjugate from the adjuvant-immunogen depot (Hurn and Chantler, 1980, Langer,

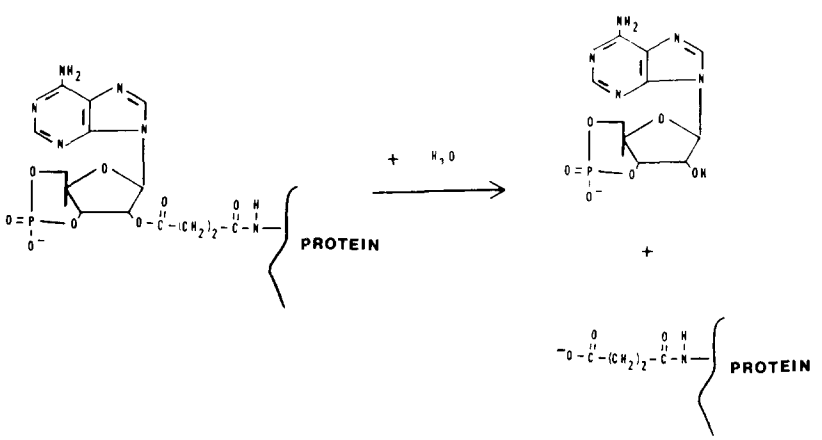

Fig 1 Hydrolysis of the O^2-ester linkage of SCAMP in protein-SCAMP conjugates

1981) First, loss of the nucleotide molecules from the protein (usually human or bovine serum albumin when preparing anti-cyclic AMP antibodies (Niswender and Midgley, 1970)) may result in less than optimal immune response and antisera with poor titer Second, the residual succinyl groups remaining linked to the carrier protein may elicit a population of antibodies specific for the residual succinyl-protein determinant sites

In this paper, we will present thin layer and high performance liquid chromatography data which clearly show that free $O^{2'}$-SCAMP is unstable in the pH range of 6 0–9 0 Furthermore, it will be shown that in vitro hydrolysis of glucose-6-phosphate dehydrogenase-hapten conjugates prepared with SCAMP and stored at neutral pH causes a progressive decrease in the inhibition of enzyme activity of such conjugates in the presence of anti-cyclic AMP antibodies Data will also be presented which point to the presence of a population of anti-succinyl-protein antibodies that likely results from the in vitro and possibly in vivo hydrolysis of the HSA-SCAMP immunogen used to elicit anti-cyclic AMP antibodies The consequences of these hydrolysis reactions with regard to the development of a new homogeneous EIA selective for cyclic AMP and to existing RIAs for cyclic nucleotides will be discussed

Materials and Methods

Reagents

The following reagents were obtained from Sigma Chemical Company, St Louis, MO $O^{2'}$-monosuccinyladenosine 3',5'-cyclic monophosphate, sodium salt (SCAMP), adenosine 3',5'-cyclic monophosphate (cyclic AMP), glucose-6-phosphate dehydrogenase Type XXIII (G-6-PDH), human serum albumin Fraction V (HSA), tris(hydroxymethyl)aminomethane (Tris), 1,3-bis[tris(hydroxymethyl)methylamino]propane (bis-Tris-propane), 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), *N*-hydroxysuccinimide (NHS), sodium azide, Freund's incomplete adjuvant, gelatin Type II from swine skin, glucose-6-phosphate, monosodium salt, β-nicotinamide-adenine dinucleotide (NAD), β-nicotinamide-adenine dinucleotide phosphate (NADP) The solvents dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were obtained from Aldrich Chemical Company, Milwaukee, WI HPLC grade methanol was obtained from Burdick and Jackson, Muskegon, MI Freund's complete adjuvant was obtained from Difco Laboratories, Detroit, MI Cellulose thin layer chromatogram sheets with fluorescent indicator were obtained from Eastman Kodak, Rochester, NY All other chemicals were reagent grade All solutions were prepared with deionized distilled water

Equipment

Ultraviolet absorption spectra were taken on a Cary Model 219 recording spectrophotometer with 1 cm path length quartz cells Activity of the conjugated enzyme was determined at 30°C using a Gilford Stasar III spectrophotometer with a thermoelectric flow-through cuvette Reaction time was monitored with a stopwatch

Finn pipettes were used for the measurement of all reagents in the assay Lyophilization of the enzyme conjugates was carried out with a Virtis lyophilizer The HPLC studies were done using a Brownlee RP-18 Spheri 5 analytical cartridge with a 3 cm precolumn of the same type, a Spectra-Physics SP8700 solvent delivery system, an SP8750 pump and injection system, a Kratos Spectroflow 773 variable wavelength detector and a Linear Model 500 chart recorder

Procedures

Conjugation reactions

Preparation of SCAMP-NHS ester 5 0 mg of the sodium salt of SCAMP was reacted with 1 7 mg EDAC and 1 0 mg NHS in a 1 2 mixture of dry DMF and dry DMSO The reaction was allowed to proceed overnight at 4°C with stirring

Preparation of SCAMP-G-6-PDH conjugates Most of the data presented in this report were obtained from 4 SCAMP-G-6-PDH conjugates designated 19-1, 19-1A, 20-2 and 20-3 For conjugates 19-1 and 19-1A, 90 μl of the solution of SCAMP-NHS ester was added in 15 μl aliquots at 10 min intervals to a solution containing 100 units (0 32 mg) G-6-PDH (which had been previously dialyzed against 2×1 0 l 0 10 M sodium bicarbonate), 1 5 mg G-6-P and 0 3 mg NADP (in 300 μl) in a 3 0 ml glass centrifuge tube with a triangular magnetic stirbar For conjugate 20-2, 180 μl and for 20-3, 135 μl of the solution of activated ester was added in the same manner The protein solution was kept at 0°C during the addition of the ester mixture, then at 4°C overnight The reaction mixtures were then dialyzed for 48 h against 4×1 0 l of the appropriate buffer The pH 8 0 buffer (used for conjugate 19-1A) was 0 10 M Tris-HCl, 0 10 M NaCl, 0 01% sodium azide For the conjugates stored at pH 7 0 (19-1 and 20-3), an initial dialysis against 2×1 0 l of 0 10 M sodium phosphate, 0 10 M NaCl was followed by dialysis against 2×1 0 l of 0 05 M bis-Tris-propane-HCl, 0 15 M NaCl, 0 01% sodium azide The solutions of conjugates were then each diluted to 2 0 ml with the dialysis buffer Spectra from 230 to 300 nm were taken in order to estimate the degree of conjugation (the moles SCAMP per mole protein) The solution of simultaneous equations using the molar absorptivities of the unconjugated enzyme and hapten together with the absorbance readings of the conjugates at 230 and 260 nm gave the following values for the SCAMP/enzyme ratio conjugate 19-1, 11, 19-1A, 9, 20-2, 22, 20-3, 17 Many other conjugates were prepared in a similar manner with similar results

Conjugates prepared in the manner described above typically retained 30–50% of their native activity (depending on the number of hapten molecules conjugated per enzyme molecule) following the conjugation procedure The specific activity of the unconjugated enzyme was 645 units/mg protein (1 unit = 1 μmol NADH/min) while those of the conjugates immediately after dialysis were 19-1, 269 units/mg, 19-1A, 291 units/mg, 20-2, 239 units/mg, 20-3, 318 units/mg Typically, the activity of the conjugates increased gradually during storage in solution, presumably due to loss of the hapten by hydrolysis There was no loss of activity in the frozen samples, either those in solution or dry

Preparation of SCAMP-HSA conjugates The immunogen was prepared by conjugating SCAMP to human serum albumin in reactions analogous to those used for the enzyme conjugates 300 μl of the ester mixture was added in 50 μl aliquots to 2 5 mg HSA in 500 μl of 0 10 M sodium bicarbonate pH 9 2 At the end of the reaction period, appropriate dialysis was carried out against PBS

Succinylation of G-6-PDH A solution of 100 units of G-6-PDH (0 32 mg), 1 5 mg G-6-P, and 0 3 mg NADP in 0 10 M sodium bicarbonate was reacted with 25 mg succinic anhydride dissolved in 400 μl DMSO overnight at 4°C The reaction mixture was then dialyzed at pH 7 0 in the manner described above This succinylated protein showed no activity and appeared to be denatured

Antiserum to cyclic AMP

Anti-cyclic AMP antiserum was obtained through the Unit for Laboratory Animal Medicine (ULAM) at the University of Michigan Medical Center Immunization of a New Zealand white rabbit was initiated by intradermal injection of 1 0 ml of an emulsion of a 1 1 mixture of Freund's complete adjuvant and a 2 mg protein/ml solution of the SCAMP-HSA conjugate previously described into 6 sites on the back of the animal The emulsion was prepared by the glass syringe double-hub connector method (Hurn and Chantler, 1980) This was followed by similar injections of the conjugate solution emulsified with Freund's incomplete adjuvant at 1, 2, 3, 5, 9 and 13 weeks Serum from a bleeding taken in the 14th week after the initial dose was used to obtain the data presented in this report Freshly prepared conjugates were used for the initial dose and those given at 2 and 5 weeks A commercial preparation of antiserum to cyclic AMP was obtained from Miles-Yeda (Rehovort, Israel) This antiserum was reportedly raised in rabbits using a similar SCAMP-HSA immunogen

Thin layer chromatography studies of the hydrolysis of SCAMP

Approximately 1–2 mg SCAMP was placed into 1 0 ml of each of the following buffers pH 6 0 (0 10 M sodium acetate), pH 7 0 (0 05 M bis-Tris-propane-HCl), pH 8 0 (0 10 M Tris-HCl), pH 9 0 (0 10 M Tris-HCl) The solutions were stored at 4°C and were examined periodically by thin layer chromatography on cellulose plates with fluorescent indicator developed with 2-propanol and 0 50 M ammonium acetate in a 3 1 ratio The SCAMP solutions were compared with underivatized cyclic AMP standards treated in the same manner

High performance liquid chromatography studies of the hydrolysis of SCAMP

To each of 6 vials containing 2 0 mg of the sodium salt of SCAMP as received from Sigma, one of the following buffers was added 0 10 M sodium bicarbonate, pH 9 0, 0 10 M Tris-HCl, pH 9 0, 0 10 M Tris-HCl, pH 8 0, 0 05 M bis-Tris-HCl, pH 7 0, 0 10 M sodium acetate, pH 6 0, 0 10 M sodium acetate, pH 4 0 For determination of concentration effects, 4 0 ml of the pH 6 0 acetate buffer was added to a seventh 2 0 mg vial of SCAMP These solutions were stored at 4°C Every second or third day for approximately 8 weeks a 10 μl aliquot was removed, diluted to 2 0 ml with a mixture of 0 01 M phosphate buffer, pH 6 5 and methanol in

a ratio of 88 12 Three 25 μl samples were injected into the sample loop in order to flush it before the final sample was allowed to enter the column The chromatography was carried out isocratically using the same 88 12 phosphate buffer-methanol mixture as the eluent

Determination of activity and inhibition of SCAMP-G-6-PDH conjugates

The activity of the conjugated enzyme was determined by measuring the increase in absorbance at 340 nm due to the appearance of NADH in an assay mixture containing 100 μl of a 1 1000 dilution of the conjugate, 5×10^{-3} mmol G-6-P, and 3×10^{-3} mmol NAD in a total volume of 800 μl The absorbance change over a 2 min period was noted after a 30 s delay to allow for temperature equilibration In order to determine the amount of antibody-induced inhibition of the conjugate, 25 μl of antiserum was substituted for a like volume of buffer The serum was allowed to incubate with the conjugate for 10 min before initiation of the reaction by the addition of substrates To test for reversal of antibody-induced inhibition by excess succinyl cyclic AMP, 100 μl of a 10^{-2} M solution was added in place of a like volume of buffer The assay buffer was 0 10 M Tris-HCl, 0 10 M NaCl, 0 01% sodium azide, pH 8 0 Conjugate dilutions were made in the pH 7 0 and 8 0 buffers described in the conjugate preparation section to which 0 1% gelatin had been added

Results and Discussion

Hydrolysis of free SCAMP

After noting several anomalies in our attempts to develop a homogeneous EIA for cyclic AMP (to be discussed below), we decided to examine the stability of the free SCAMP derivative (used in all conjugation procedures) in different buffers of varying pH Preliminary TLC and subsequently a more quantitative study using HPLC were performed as described in the experimental section The TLC study showed that a spot with an R_F value corresponding to that for underivatized cyclic AMP appeared within one day when succinyl-cyclic AMP was stored at pH 8 0 or 9 0, after 4 days at pH 7 0, and after 22 days at pH 6 0 After storage for 3 weeks at pH 9 0 and 4 weeks at pH 8 0, the spot corresponding to the original SCAMP was no longer visible

The results of the HPLC study are summarized in Fig 2 The data points in Fig 2 represent the peak height due to free cyclic AMP as a percent of the sum of the peak heights due to both the starting material (SCAMP) and the hydrolysis product (cyclic AMP) The results of the HPLC study strongly confirm the qualitative TLC data It is clear that the rate of hydrolysis of SCAMP to cyclic AMP and succinic acid increases dramatically with increasing pH

However, in addition to the effect of pH, the nucleophilicity of the buffer also appears to play a role in this process As can be seen in Fig 2, the rate of appearance of free cyclic AMP is much faster in Tris at pH 8 0 than in carbonate at pH 9 0 and much faster in bis-Tris at pH 7 0 than in acetate at the more acidic pH

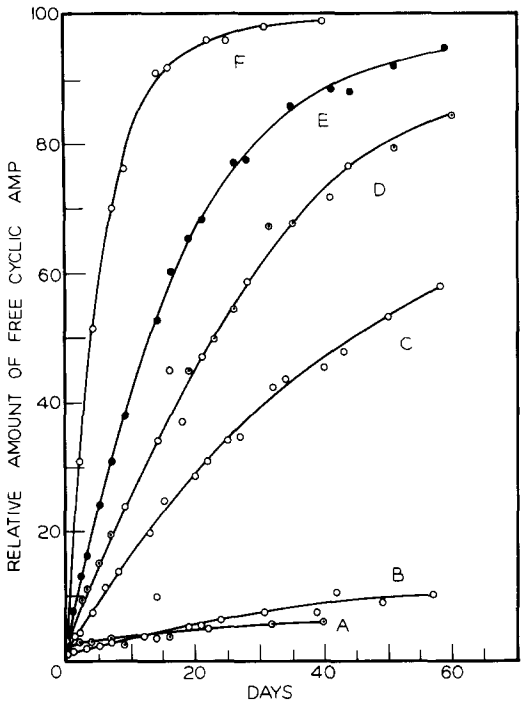

Fig 2 Rate of appearance of free cyclic AMP as the result of hydrolysis of SCAMP during storage at 4°C in A pH 4 0 acetate buffer, B pH 6 0 acetate buffer, C pH 7 0 bis-Tris-HCl buffer, D pH 9 0 carbonate, E pH 8 0 Tris-HCl, F pH 9 0 Tris-HCl

of 4 0 or 6 0 It appears that the free amino groups in the Tris type buffers help to catalyze the cleavage reaction more so than the carboxyl groups in carbonate and acetate regardless of pH

Hydrolysis of SCAMP in protein-SCAMP conjugates

Upon linking SCAMP to the enzyme glucose-6-phosphate dehydrogenase for ultimate use in a homogeneous EIA for cyclic AMP, we noted a marked decrease over time in the ability of anti-cyclic AMP antiserum (either that raised at the University of Michigan or Miles commercial lot) to inhibit the catalytic activity of these conjugates Fig 3 shows the decreases observed for the inhibition of SCAMP-G-6-PDH conjugates stored in pH 7 0 and pH 8 0 buffers over a period of 4 weeks at 4°C and at −20°C Conjugate 19-1, stored at pH 7 0, was initially inhibited 85% by excess anti-cyclic AMP antibody 3 days after conjugation Inhibition decreased to 53% after 4 weeks at 4°C and to only 33% when the same conjugate was stored at −20°C The decrease in inhibition of conjugate 19-1A stored at pH 8 0 was even more dramatic From an initial value of 77% after 3 days it decreased to only 29% inhibition after 4 weeks at 4°C and to only 11% after the same time at −20°C A host of similar conjugates were also examined and all showed a similar decrease of inhibition with prolonged storage To further demonstrate the hydrolytic loss of

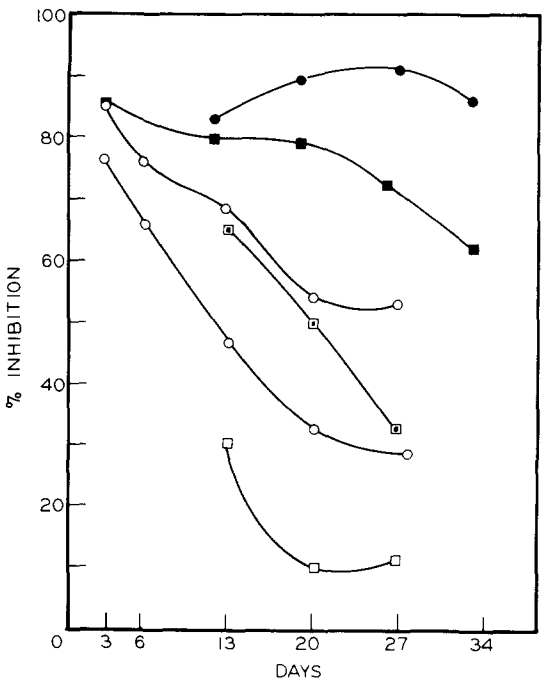

Fig 3 Decrease in antibody-induced inhibition of SCAMP-G-6-PDH conjugates stored in solution at pH 7 0 (○) at 4°C and (□) at −20°C, pH 8 0 (○) at 4°C and (□) at −20°C and comparison of antibody-induced inhibition of conjugate 20-2 stored in pH 7 0 buffer at 4°C (■) with that observed in the same conjugate after lyophilization (●)

cyclic AMP molecules from the enzyme-SCAMP conjugates, after the 4-week-storage period these conjugates were redialyzed against 2 × 1 0 l of pH 7 0 bis-Tris-propane-HCl (conjugate 19-1) or pH 8 0 Tris-HCl (conjugate 19-1A) Data obtained from UV spectra for these conjugates after dialysis treatment are shown in Table I The UV absorbance at 260 nm falls dramatically presumably due to a loss of the nucleotide chromophore (absorption maximum for cyclic AMP = 259 nm (Fasman, 1976)) The decrease in the ratio of the absorbances at 260 nm and 230 nm (260/230), is a more

TABLE I

COMPARISON OF ABSORBANCE (A) AT 260 AND 230 nm AND THE RATIO OF THE 2 ABSORBANCES FOR CONJUGATES 19-1 AND 19-1A IMMEDIATELY AFTER PREPARATION WITH THAT FOUND AFTER REDIALYSIS 4 WEEKS LATER

Conjugate	Initial			Dialysis after 4 weeks			Decrease
	A_{260}	A_{230}	Ratio	A_{260}	A_{230}	Ratio	
19-1	0 436	1 092	0 399	0 098	0 495	0 206	48%
19-2	0 364	1 030	0 353	0 109	0 530	0 206	42%

valid measure of the degree of hydrolysis, since this value normalizes for any decreases in the protein concentrations due to non-specific adsorption and dilution effects (i e , enzyme has significant absorption at 230 nm whereas cyclic AMP does not)

Although storage of the enzyme-SCAMP conjugates at −20°C does not slow the rate of hydrolysis, lyophilization of the conjugates soon after preparation does stop the decomposition process For example, when a new series of conjugates (similar to 19-1 and 19-1A) was lyophilized immediately after preparation, these conjugates exhibited essentially no loss in the ability of anti-cyclic AMP antibodies to inhibit catalytic activity over 4 weeks when stored at −20°C, see Fig 3 We have since accumulated data for lyophilized conjugates showing no loss of activity or ability to be inhibited during storage for over 1 year

Evidence for hydrolysis of HSA-SCAMP immunogen resulting in formation of anti-succinyl-protein antibodies

Just as the SCAMP residues are hydrolyzed in SCAMP-G-6-PDH enzyme conjugates, so too can this hydrolysis take place in the HSA-SCAMP conjugates used as immunogens for formation of anti-cyclic AMP antibodies Such hydrolysis can occur in vitro while storing the immunogen in solution prior to immunization and subsequent booster injections as well as in vivo after the immunogen has been introduced into the animal Thus, in vivo, the immunogen will have both SCAMP-protein determinant sites and some succinyl-protein sites While it may be possible that anti-succinyl-protein antibodies are elicited even in the absence of hydrolysis (the effect of bridging group recognition has been cited by many researchers, Cailla et al , 1973, Harper and Brooker, 1975) we believe that the presence of residual succinyl-protein sites remaining after hydrolysis of the hapten is likely to result in a larger population of such antibodies which have a greater affinity for this structure than those elicited to the bridging group with hapten attached

Immunological evidence for the formation of such an anti-succinyl-protein population of antibodies was gained from 2 separate studies First, a pseudoconjugate of succinylated G-6-PDH was prepared (use of a large excess of succinic anhydride resulted in loss of enzymatic activity) and tested for immunoreactivity with antiserum obtained in response to immunization of rabbits with a SCAMP-HSA conjugate A ring test was positive within 30 min and precipitin formation took place overnight A positive precipitin test was also observed with the commercial anti-cyclic AMP serum obtained from Miles-Yeda Laboratories Appropriate blank precipitin tests in which succinylated G-6-PDH was mixed with normal rabbit serum yielded no noticeable precipitin formation

Second, we also evaluated the ability of excess free SCAMP to reverse the inhibitory effect of the anti-cyclic AMP antiserum on SCAMP-G-6-PDH conjugates similar to those described in Fig 3 Complete reversal of antibody-induced enzyme inhibition would be expected if the only population of antibodies effectively inhibiting the enzyme-SCAMP conjugate were binding to the cyclic AMP residues attached to the enzyme Such reversal would be required if a useful homogeneous EIA for cyclic AMP were to be developed However, in addition to the progressive decrease

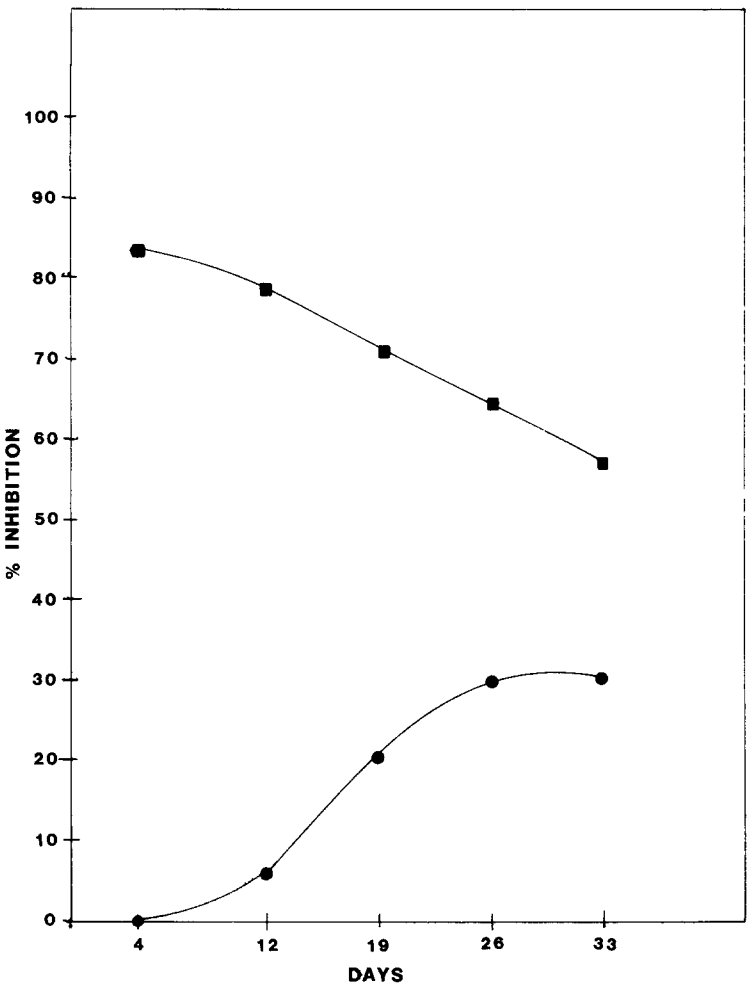

Fig 4 Increase in non-reversal of antibody-induced inhibition of SCAMP-G-6-PDH conjugate stored in pH 7 0 buffer Antibody only (■) Antibody $+10^{-2}$ M SCAMP (●)

in inhibition of enzymatic activity of the conjugates in the presence of antiserum to cyclic AMP, we observed that the ability of free cyclic AMP to completely reverse this inhibition also progressively decreased, as shown in Fig 4 It is this decrease in the reversibility of inhibition in the presence of excess SCAMP that rules out the simple loss of succinyl cyclic AMP bound to the protein through unstable linkages to serine, threonine or tyrosine Hydrolysis of SCAMP from these residues would certainly result in loss of inhibition of the enzymatic activity by the anti-cyclic AMP antibody but does not explain the decreasing reversibility of the remaining inhibition by excess SCAMP which was observed

Several possibilities were investigated to account for the non-reversal observed Redialysis of the stored conjugate to remove hydrolyzed cyclic AMP that could

occupy binding sites of the antibody had no effect on inhibition or reversibility of inhibition This suggests the presence of a second population of antibodies in the serum with the ability to bind to the conjugate and inhibit its activity even in the presence of excess SCAMP (10^{-2} M) The possibility that hydrolysis of the cyclic phosphate group had occurred during immunization was also examined If this had occurred, anti-AMP antibodies would have been elicited This was ruled out by the observation that the addition of AMP to the assay mixture had no effect on the inhibition or reversal of inhibition of the conjugate activity However, the addition of a succinylated G-6-PDH pseudoconjugate (no enzymatic activity) to the assay

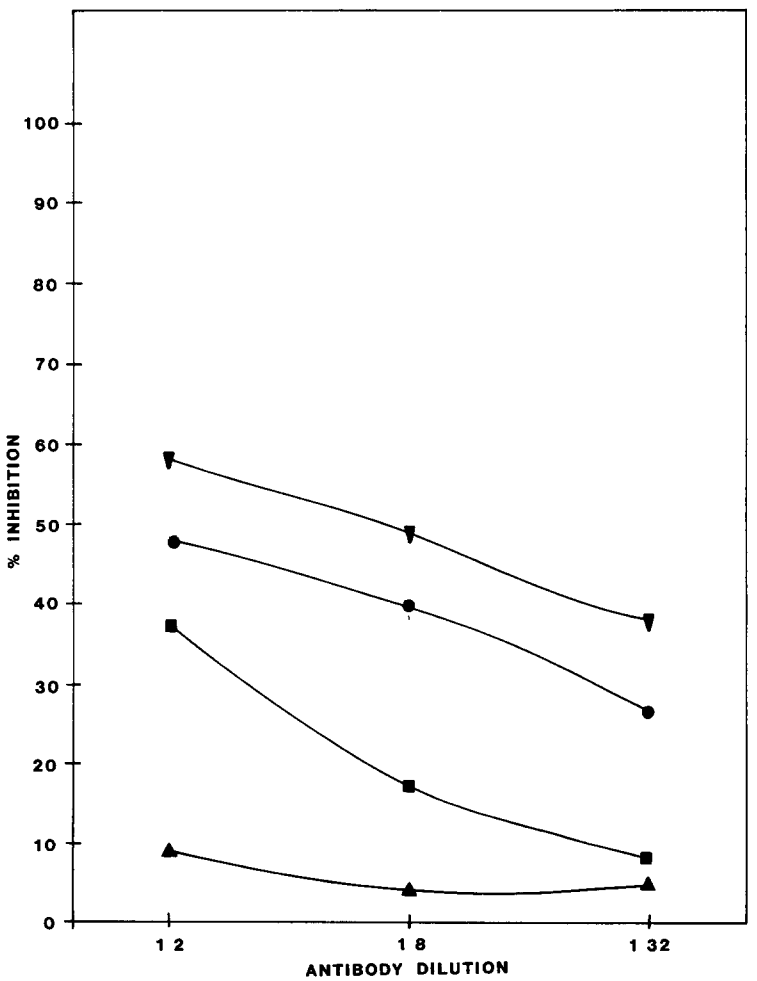

Fig 5 Effects of dilution of antiserum and of inclusion of succinylated protein on non-reversibility of antibody-induced inhibition of SCAMP-G-6-PDH conjugates stored in pH 7 0 buffer Antibody only (▼) Antibody + succinylated protein (●) Antibody + 10^{-2} M SCAMP (■) Antibody + succinylated protein + 10^{-2} M SCAMP (▲)

mixture did result in reversal of the inhibition remaining in the presence of excess SCAMP (see Fig 5) This confirms that the site of hydrolysis is at the $O^{2'}$ position and that an interfering population of antibodies with apparent specificity for the succinyl-protein structure is present Such behavior suggests that as cyclic AMP molecules are lost from the enzyme-SCAMP conjugate with time, the fraction of total inhibition due to anti-cyclic AMP specific antibodies in the antiserum decreases, whereas the fraction of total inhibition resulting from a secondary antibody population directed toward residual succinate-protein sites increases

The observations described above provide strong evidence that the ester linkage of $O^{2'}$-monosuccinyl cyclic AMP (SCAMP) is unstable when this derivative is free in neutral solution or conjugated to a protein via an amide linkage Thus, in vitro and in vivo hydrolysis of SCAMP-HSA immunogens can lead to the production of antibodies more specific for protein-bound succinyl groups than those elicited toward the bridging group in the absence of hydrolysis In view of the dilute and/or low affinity nature of this population of antibodies in anti-cyclic AMP serum, it is not surprising that the presence of this secondary antibody population has never been observed in existing RIAs or heterogeneous EIAs of cyclic AMP (Meyerhoff and Rechnitz, 1979, Joseph and Guesdon, 1982) This is because, in such assays, antisera are often diluted on the order of 1 10,000–1 50,000 (Steiner et al , 1969, 1972) and any binding of hydrolyzed labels by secondary antibodies goes undetected Further, in RIAs the continuous decay of radioactivity with time may partially cover up decreases in the binding of the label due to hydrolysis Indeed, it is likely that the iodinated tyrosine methyl ester-SCAMP molecules used as tracers in RIAs of cyclic AMP will hydrolyze at the O^2 ester linkage resulting in less 'bindable' radioactivity with time It is also likely that the resulting tyrosine methyl ester of succinate will not bind nearly as well to anti-succinyl-protein antibodies as do succinyl-protein sites (i e in protein conjugates the succinyl group is most often linked to lysine residues not tyrosine residues) Thus, free succinate has only minimal effect on the RIA assays of cyclic AMP (Harper and Brooker, 1975)

The problems of hydrolysis and anti-succinyl antibody formation have a much greater effect on homogeneous EIAs, where each enzyme-SCAMP conjugate is required to be surrounded by a large number of anti-cyclic AMP antibodies in order to induce substantial homogeneous inhibition of the conjugates (Rowley et al , 1975) Consequently, in this case, antisera cannot be diluted nearly as much as when being used in RIAs or even in heterogeneous EIAs, and problems regarding non-reversible binding of the label can often occur Lyophilization of the enzyme-SCAMP conjugates immediately after preparation overcomes this problem and allows for the development of a useful homogeneous EIA for cyclic AMP provided that the enzyme conjugates are reconstituted immediately prior to cyclic AMP assays

Conclusions

We believe that hydrolysis of succinyl derivatives of haptenic compounds at neutral pH can become a serious concern when developing antiserum towards the

haptenic groups as well as in the development of certain immunoassays for these molecules While we have focused only on the case of SCAMP and assays for cyclic AMP based on this derivative, it should be clear that succinyl derivatives of steroids and other drugs used routinely to prepare immunogens may also be prone to this hydrolysis reaction Thus, it is suggested that derivatives containing linkages less subject to hydrolysis, such as amides, ethers and thio-ethers be developed for use where long-term stability in solution is essential, such as in the preparation of hapten-protein immunogens and other conjugates (e g , enzyme-hapten conjugates)

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