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14. We thank A. Kadous for technical assistance with the adenosine triphosphatase assays. Supported by the College of Agricultural and Life Sciences, University of Wisconsin, and by the following research grants: grant FD-00250 from the U.S. Public Health Service; grant 2-04-4200 from the Department of Natural Resources, State of Wisconsin; and grant 133-4957 from the Brittingham Trust Fund through the Marine Studies Center, University of Wisconsin.

4 February 1972; revised 17 April 1972

Morphine-3-Succinyl–Bovine Serum Albumin: An Immunogenic Hapten-Protein Conjugate

Abstract. *Morphine-3-hemisuccinate was synthesized by reaction of morphine with succinic anhydride. This compound was conjugated to bovine serum albumin by the mixed anhydride method, and the degree of conjugation was determined by base hydrolysis of the conjugate, extraction, and measurement of free morphine. An average of 6.5 molecules of morphine were conjugated to each molecule of protein. Eleven rabbits immunized with varying doses of the conjugate were producing antibody 8 weeks later, as determined by a modification of the ammonium sulfate method, which measures primary binding of antigen by antibody.*

Antibodies specific for physiologically active haptens can have specific antagonistic effects in vivo (*1*). In immunologic assay systems for the detection of small quantities of morphine and related opium alkaloids in biological fluids (*2–4*), the 3-*O*-carboxymethyl derivative of morphine coupled to bovine serum albumin (BSA) has been used as antigen. Since the presence or absence of antagonism in vivo may hinge on either differences in antibody affinity or on the ability of antibody to recognize critical regions in the hapten structure, it seems important to study the antibody response to different types of morphine-protein conjugates.

We have prepared a succinic acid ester of morphine which conjugates to BSA forming stable, easily definable hapten-protein conjugates. Evidence for the antigenicity of morphine-3-hemisuccinate (M-3-HS) conjugated to BSA is presented.

Morphine-3-hemisuccinate (*5*) was prepared by heating morphine with three equivalents of succinic anhydride in pyridine for 4 hours (Fig. 1). The pyridine was evaporated at reduced pressure, the residue was washed five times with hot ethanol, and the residue was recrystallized twice from 60 percent ethanol, yielding crystals melting at 237° to 239°C (with decomposition). Thin-layer chromatography (TLC) was performed in a solvent system of ethyl acetate, methanol, and ammonium hydroxide (85:10:5). The crystalline product would not migrate in this solvent system, while morphine migrated with an R_F of 0.3. A ferric chloride color test for free phenol was negative. The infrared spectrum of the product showed bands at 1730 cm^{-1} (ester carbonyl) and between 1550 to 1660 cm^{-1} (carboxylate). The mass spectrum showed: m/e, 385(M^+); 341 (M-CO_2); 285 (M-$COCH_2CH_2CO_2$); 268 (M-$COCH_2CH_2CO_2$-OH). Elemental analysis showed C, 65.17; H, 6.19; and N, 3.52 percent; calculated values for M-3-HS ($C_{21}H_{23}O_6N$) are C, 65.44; H, 6.02; and N, 3.63 percent.

The M-3-HS was conjugated to BSA by the mixed anhydride method (*6*). To 25 ml of dioxane we added 0.300 g (0.744 mmole) of M-3-HS, 0.097 ml (0.744 mmole) of isobutyl chloroformate, and 0.178 ml (0.744 mmole) of tributylamine. The mixture was stirred for 30 minutes with the temperature maintained below 20°C (Fig. 1). Crystalline BSA (Pentex) (0.5210 g; 0.0074 mmole) was dissolved in 100 ml of water, and 100 ml of dioxane (spectral grade) was added slowly with stirring. The solution of BSA in water and dioxane was initially *p*H 5, which was adjusted to *p*H 8.5 with 1*N* NaOH. The solution became cloudy at approximately *p*H 6 and then cleared above *p*H 7. The BSA solution was cooled to 5°C, and at the end of 30 minutes the mixture of M-3-HS and isobutyl chloroformate was added. An additional 25 ml of water was added

Table 1. The effect of different doses of M-3-HS-BSA on serum antigen-binding capacity and relative antibody affinity. The antigen was emulsified in complete Freund's adjuvant and injected subcutaneously into rabbits on day 0 and day 49. The antigen-binding capacity (ABC) is expressed as the number of picomoles of morphine bound per milliliter of undiluted antiserum when 88.0 pmole of [^{14}C]morphine was used. The effect of dilution (ED), a measure of relative antibody affinity (*9*), is (ABC at 8.8 pmole [^{14}C]morphine/ABC at 88.0 pmole [^{14}C]morphine) × 100; Tr, trace; 0, no detectable binding.

Conjugate immunizing dose (mg)	5 weeks		8 weeks		13 weeks	
	ABC	ED	ABC	ED	ABC	ED
0.5	556	18.0	3733	48.1	5064	65.7
	1088	29.8	5556	63.8	2402	48.8
	1186	8.2	2199	50.0	506	51.7
	Tr		1186	61.0	1013	65.7
Mean	708	18.7	3806	55.7	2246	58.0
5.0	535	21.9	4138	53.1	1187	83.7
	Tr		2373	69.8	1302	88.9
	1273	39.3	13892	61.5	10998	51.9
	0		660	66.7	1070	66.2
Mean	452	30.6	5266	62.8	3639	72.5
50.0	0		Tr		0	
	0		318	61.8	0	
	Tr		1447	54.0	Tr	
Mean	0		588	57.9	0	

to keep the ratio of water to dioxane constant at 1:1. The mixture was stirred for 4 hours at 5°C, and the *p*H was maintained at 8.5 with 1*N* NaOH.

After 4 hours the reaction mixture was concentrated to 25 ml by pressure dialysis against water. Gel filtration with Sephadex G-50 separated the remaining unreacted M-3-HS from the conjugate.

The number of molecules of morphine conjugated per molecule of BSA was determined by hydrolyzing the purified conjugate under basic conditions and measuring spectrophotometrically the concentration of free morphine generated. Specifically, 100 mg of M-3-HS-BSA was dissolved in 10 ml of water. The solution was cooled to 5°C; the *p*H was adjusted to 12.5 with 1*N* NaOH, and then the solution was stirred at 5°C for 24 hours. Under such conditions the ester linkage connecting the morphine nucleus to the succinic acid moiety was selectively hydrolyzed leaving most of the amide bonds intact. As a control 100 mg of crystalline BSA was subjected to the same procedure.

The hydrolysis mixtures of M-3-HS-BSA and unconjugated BSA were chromatographed on a Sephadex G-25 column equilibrated with phosphate-buffered saline. The BSA emerged with the void volume. A second peak having significant optical density at 280 nm was observed only with hydrolyzed samples of M-3-HS-BSA. Fractions containing the second peak of optical density from the M-3-HS-BSA sample and the comparable fractions obtained with the unconjugated BSA were pooled, adjusted to *p*H 9.1 with 20 ml of sodium borate buffer (*p*H 9.3), and extracted six times with a chloroform-isopropanol (3:1) solution (*7*). The organic layers were separated, evaporated to dryness, and redissolved in 10 ml of methanol. The optical densities of both methanol solutions were then read at 285 nm. The difference between the two optical densities was taken to be proportional to the amount of morphine present. The material from the second peak and morphine had essentially identical behavior in TLC and had the same ultraviolet spectra between 210 and 320 nm and the same mass spectra. Based upon the amount of morphine recovered from the hydrolyzate, the extent of conjugation was 6.5 molecules of morphine per molecule of BSA.

Eleven New Zealand white rabbits were inoculated subcutaneously with

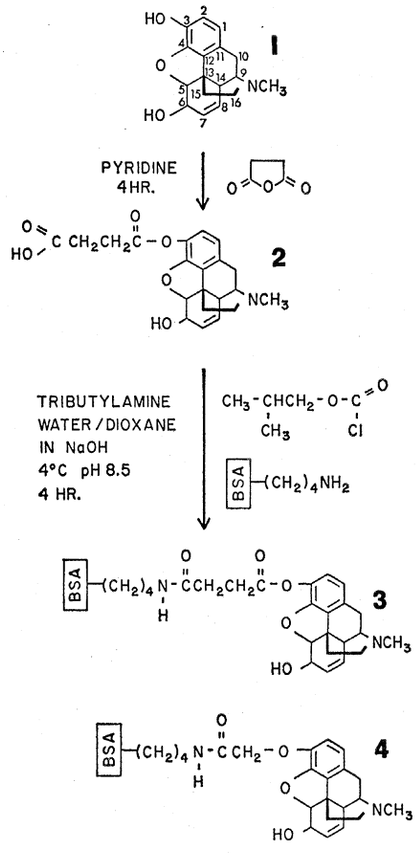

Fig. 1. Preparation of morphine-3-hemisuccinate and conjugation to BSA. Structure **1** is morphine; structure **2** is morphine-3-hemisuccinate (M-3-HS); structure **3** is M-3-HS-BSA; and structure **4** is 3-*O*-carboxymethylmorphine–BSA, the conjugate described by Spector and Parker (*2*).

either 0.5, 5, or 50 mg of M-3-HS-BSA emulsified in complete Freund's adjuvant. The antigen-binding capacity of the serums was determined by a modification (*8*) of the ammonium sulfate method (*9*). By week 8 antibody was detected in the serums of the 11 animals (Table 1). Significantly lower antigen binding was found in serums from the rabbits immunized with 50 mg of conjugate. Also measured was the effect of antigen dilution on the antigen-binding capacity, which provides a qualitative measure of antibody affinity (*9*). The results indicate that the binding strengths increased gradually and appeared to be maximal after 5.0 mg had been used for immunization. The specificity of antigen binding was demonstrated by competition studies with the addition of unlabeled morphine or other opiates prior to that of ^{14}C-labeled morphine. Drug concentrations which inhibited 50 percent of the binding of [^{14}C]morphine (I_{50}) were as follows: morphine, 110 pmole/ml; heroin, 100 pmole/ml; codeine, 160 pmole/ml; naloxone, > 1000 nmole/ml; and meperidine, 80 nmole/ml.

Both morphine-3-hemisuccinate and 3-*O*-carboxymethylmorphine, the derivative used by Spector and Parker (*2*), are assumed to form amide linkages with free amino groups on the BSA molecule (Fig. 1). For 3-*O*-carboxymethylmorphine the bridge between the amide nitrogen and the morphine nucleus consists of three atoms (carbonyl of the amide bond, the methylene carbon, and the oxygen of the ether bond). The bridge of the hemisuccinate conjugate is composed of five atoms (carbonyl of the amide bond, two methylene carbons, and the ester linkage). Differences in bridge length and degrees of freedom places the hapten moieties of each conjugate in different steric environments. This may result in production of antibodies of differing affinity and specificity (*10*). A comparison of the above inhibition data with those previously published (*4, 11*) indicates that such differences may be present.

Evidence has been presented (*12*) that the degree of conjugation of a hapten to its carrier can affect the immunologic response to the hapten. This should provide another factor to be considered in the immunopharmacology of morphine, and it is therefore essential that the number of hapten molecules per molecule of carrier be estimated with reasonable accuracy. The use of alkaline hydrolysis to estimate the degree of morphine conjugation appears to be a sensitive procedure, and its advantage is that free morphine can be extracted and measured directly.

Thus elucidation of the effects in vivo of antiserum on the pharmacologic actions of morphine may hinge on an understanding of how different species of antibody recognize the drug molecule. The M-3-HS-BSA represents a stable, easily definable hapten-protein conjugate. The antibody response to the morphine moiety within the steric environment of this conjugate should provide additional information concerning the antigenic determinants on the drug molecule.

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13. Supported by PHS training grant 5 TO5 GMO 1939-04 (MSTP) from the University of Chicago.

21 December 1971; revised 12 March 1972

Crystalline Fraction I Protein: Preparation in Large Yield

Abstract. *About 1 milligram of twice-recrystallized fraction I protein of constant specific ribulose diphosphate carboxylase activity per gram of leaves (fresh weight) has been obtained from each of seven different species of* Nicotiana *and 14 reciprocal, interspecific F_1 hybrids. Crystals are produced from homogenates that have only been centrifuged to remove particulate matter.*

Two curious properties of crystalline fraction I protein (*1*) isolated from *Nicotiana tabacum* leaves have been described. (i) Storage at 0°C caused substantial loss in specific ribulose diphosphate carboxylase activity (*2*), which was entirely regained by heat treatment, whereas the activity remained unchanged during prolonged storage at 25°C. (ii) Protein in the presence of ribulose-1,5-diphosphate was extremely soluble (> 100 mg/ml), whereas in the absence of ribulose-1,5-diphosphate and the presence of Mg^{2+} and HCO_3^-, the enzyme was extremely insoluble (< 1.0 mg/ml) (*3*). The latter property, in particular, provided the basis for the following method which has also been successfully used elsewhere for isolating the protein from *N. tabacum* leaves by crystallization. In our laboratory, the method has been successful with seven additional species of *Nicotiana* as well as 14 reciprocal, interspecific *Nicotiana* F_1 hybrids. The general procedure is as follows.

Grind to a smooth paste 15 g of demidribbed leaves with 15 ml of ice-cold buffer A (0.05*M* tris-HCl, *p*H 7.4; 1.0*M* NaCl; 0.001*M* EDTA; 0.002*M* $MgCl_2$; 0.08*M* β-mercaptoethanol) using a pestle and a mortar surrounded by ice. Filter paste through cheesecloth and Miracloth and squeeze to obtain juice, which is centrifuged at 17,000*g* for 5 minutes. Remove any floating material; decant the supernatant and centrifuge it at 17,000*g* for 30 minutes. The supernatant from *N. tabacum* will still be green; from other species and hybrids, it is usually green to yellow. The volume is about 22 ml.

At room temperature, pass 22 ml of the supernatant through a G-25 Sephadex column (1 by 50 cm) previously equilibrated with buffer B [0.025*M* tris-HCl, *p*H 7.4; 0.20*M* NaCl; 0.0005*M* EDTA (no Mg; no mercaptoethanol)] and elute with this same buffer. Test successive fractions of eluate for the presence of protein by testing for a precipitate when trichloroacetic acid (TCA) is added. Collect the first 12 ml of eluate after the first TCA precipitate appears. Concentrate 12 ml to 4 ml by placing the eluate in collodion dialysis bags and subjecting them to reduced pressure or to pressurized membrane filtration. Remove the solution from the bag or membrane with the use of 0.5 to 1 ml of 0.2*M* NaCl, if necessary, to ensure that the concentrated protein layer on the wall of the bag or membrane is also removed, and centrifuge to remove any undissolved material, particularly green aggregates. Using solutions of 1*M* each, add enough to the supernatant to make the concentration of $NaHCO_3$ 0.02*M* and that of $MgCl_2$, 0.003*M*. Incubate for 15 minutes at 30°C. Transfer to a collodion dialysis bag and submerge the conical end of the bag to a depth of about 5 cm into 200 ml of buffer C (0.025*M* tris-HCl, *p*H 7.4; and 0.0005*M* EDTA) contained in a 250-ml Erlenmeyer flask. Leave undisturbed either at room or ice-box temperature, the latter seeming to accelerate the formation of fraction I protein crystals, which usually appear overnight and which will have the appearance in the bag as shown in Fig. 1. Remove mother liquor by a Pasteur pipette. Scrape the crystals off the wall, suspend them in buffer C, and transfer them to a small centrifuge tube. Collect the crystals by allowing them to settle or by gentle centrifugation; in either case remove the wash liquid.

Wash again with fresh buffer C. Add 1 ml of buffer B to dissolve crystals. Centrifuge again to remove any undissolved material. Transfer solution to a collodion bag and dialyze against buffer C as before. Crystals will appear in a few hours, maximum yield is obtained after about 15 hours and should be around 1

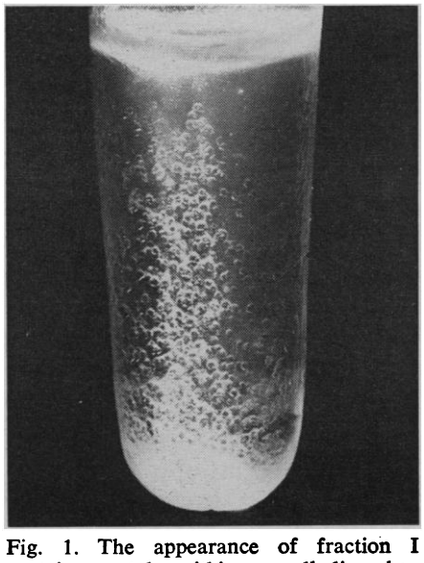

Fig. 1. The appearance of fraction I protein crystals within a collodion bag after dialysis for 16 hours. The maximum diameter of the bag is 1.5 cm.

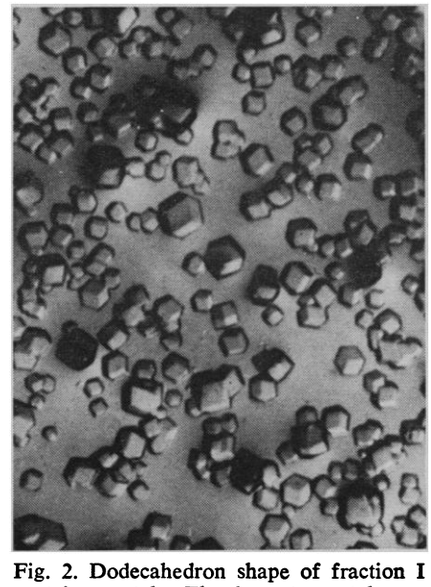

Fig. 2. Dodecahedron shape of fraction I protein crystals. The largest crystals are more than 0.2 mm in diameter.