percent level. For purposes of analysis a second line was fitted to the data without regard to origin: $D_T = 0.106 + 0.886\ C$. Parallel analyses were performed with this curve and the one forced through the origin. Differences among levels of treatment (applied centrifugal force) were found to be significant for both lines. When the linear component of the treatment term was tested separately, it was found to be significant for either line, while the remaining higher order components (or nonlinear variation) were found to be nonsignificant and could be considered as residual scatter. The slope of either line was nonsignificantly different from a value of 1. The intercept value was nonsignificantly different from 0. It must be mentioned that the variances were found to be moderately heteroscedastic, increasing somewhat with increasing magnitude of applied centrifugal force; the "maximum-*F*-ratio" test [H. A. David, *Biometrika* **39**, 422 (1952)] indicated significance at the 5 percent level. This condition somewhat reduces the accuracy of the unweighted regression parameters calculated, but analysis of variance is considered sufficiently robust to be still reliable here.

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2 March 1964

Antibodies to Bradykinin and Angiotensin: A Use of Carbodiimides in Immunology

Abstract. *Antibodies to bradykinin and angiotensin have been produced in rabbits by the use of conjugates containing albumin and the hapten, covalently bound. The use of water-soluble carbodiimide reagents provided an easy and rapid method of synthesis of the antigenic conjugates.*

Formation of antibodies to substances of low molecular weight can be stimulated by injecting compounds containing the small molecule conjugated to proteins. Conjugation has usually been achieved by diazotization or other organic syntheses (*1*). We have used water-soluble carbodiimides (*2, 3*), recently developed coupling reagents, to synthesize immunogenic conjugates of protein and biologically active small polypeptides.

Carbodiimides can couple compounds containing many types of functional groups, including carboxylic acids, amines, phosphates, alcohols, and thiols, with the formation of amides, esters, and so forth (*4, 5*). The coupling probably proceeds in at least two steps, as illustrated in reactions *A* and *B* of Fig. 1, for the postulated coupling of hapten to protein through an amide linkage (*4, 6*).

Carbodiimides also can add to carboxylic acids, and, by rearrangement, form a stable *N*-substituted urea. This is illustrated in reactions *A* and *C* of Fig. 1. This addition would yield, in the case of albumin, a substituted urea bound to carboxyl groups of the protein.

It is likely that both products, the hapten-protein complex and the substituted urea-protein complex, were formed during synthesis of the immunogens described in this report. Thus, two "foreign" antigenic determinants were added to the carrier protein, and antibodies to both might have been formed. To minimize the complications of antibodies to the substituted urea, two different carbodiimides were used. The animals were immunized with a hapten-protein conjugate synthesized by the use of one carbodiimide, and their serums were tested with conjugates synthesized by the second carbodiimide.

The two carbodiimides (Fig. 2) were reagent I, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride ("Ethyl CDI") (*7*), and reagent II, 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-*p*-toluenesulfonate ("Morpho CDI") (*8*). The carrier protein was rabbit serum albumin (RSA), and the haptens were synthetic bradykinin and synthetic angiotensin (*9*).

Approximately 10 mg of rabbit serum albumin and 20 mg of bradykinin (or other hapten) were dissolved together in 0.5 ml of water. To this mixture was added 0.25 ml of water containing 100 to 200 mg of freshly dissolved carbodiimide reagent I or II. The reaction was permitted to proceed with gentle agitation at room temperature for 5 to 30 minutes. The unadjusted *p*H of the reaction mixture was 6 to 8. The reaction was terminated by dialysis against water for 24 hours. Indirect evidence of successful conjugation of the reactants to albumin was sometimes provided by the formation of precipitates or colloidal suspensions, probably caused by changes in the solubility of albumin when substituents were added. However, formation of visible precipitates did not always follow the formation of conjugates. When precipitates formed, the granular and soluble materials were used together for immunization.

Amino acid analysis of the antigen conjugate of bradykinin and albumin for lysine, histidine, and arginine was performed by the procedure outlined by Spackman, Stein and Moore (*10*). The results are summarized in Table 1. Since

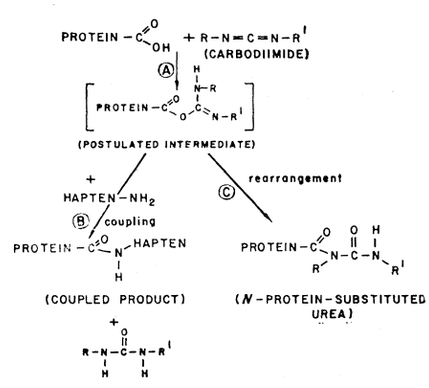

Fig. 1. Probable mechanisms of protein-hapten-carbodiimide reactions.

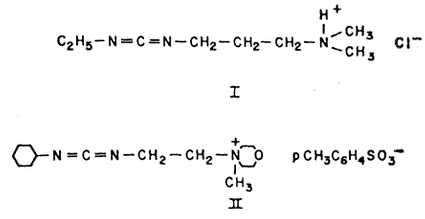

Fig. 2. Structural formulas of two water-soluble carbodiimides.

each molecule of bradykinin contains two arginine residues, but no histidine or lysine, the presence of bradykinin in a protein conjugate is indicated by an excess of arginine to histidine or to lysine as compared to the unconjugated protein. Two such conjugates, the products of separate syntheses, contained 12 moles of bradykinin per mole of albu-

Table 1. Calculation of the amount of bradykinin conjugated to albumin by reaction with carbodiimide reagent. Calculated moles of bradykinin per mole of complex was 12. (Bradykinin contains two residues of arginine but no lysine or histidine.)

Specimen	Arg / Lys*	Arg / His*	Molar excess of arginine in complex
	Experiment 1		
Rabbit serum albumin†	21	19	
Albumin-bradykinin complex	44	44	23–25
	Experiment 2		
Rabbit serum albumin†	21	21	
Albumin-bradykinin complex	45	45	24

* Native rabbit serum albumin contained 55 lysine residues and 21 histidine residues per mole. Bradykinin content calculated from arginine : lysine and arginine : histidine ratios.
† Albumin treated with "Ethyl CDI" in absence of bradykinin.

min. A repetition of the analysis after dialysis of the complex against 1.0*M* NaCl and 0.1*M* phosphate at *p*H 5.8 and *p*H 8.5 showed the same content of bradykinin, suggesting that it was bound to the albumin by covalent bonds.

When a conjugate of bradykinin and albumin was synthesized with modified albumin in which most of the amino groups were blocked by reaction with methyl acetimidate, less bradykinin was bound in the final complex (*11*). This would suggest that the original conjugate contained bradykinin bound in part by way of amino groups of the protein. Both albumin and bradykinin contain carboxyl, amino, and serine hydroxyl groups which may have combined in several ways to form amide and ester bonds between the two molecules.

The antigenic compound synthesized with "Ethyl CDI" was used to immunize rabbits. Approximately 3 mg of conjugate suspended in complete Freund adjuvant was injected into the toe-pads and leg muscles of albino New Zealand rabbits. After 3 weeks, 1 to 2 mg of the conjugate in adjuvant was readministered intraperitoneally. Serums collected 1 week after the second injection yielded specific antibodies. The titer of complement-fixing antibodies increased with subsequent intraperitoneal immunizations.

The antigen conjugate synthesized with "Morpho CDI" reagent was used in the experiments with antiserums in vitro. Complement-fixing antibodies to the conjugate were measured by the method of Wasserman and Levine (*12*). Complement-fixing antibodies to the conjugate containing bradykinin were successfully produced in all three of the rabbits injected with albumin-bradykinin (RSA-bradykinin). Two rabbits received RSA-angiotensin injections, and both produced specific antibodies (*13*).

Figure 3*A* depicts a complement-fixation curve with one antibradykinin serum and the RSA-bradykinin compound. The potency of the three antiserums varied from 1 : 200 to 1 : 1000. As shown, conjugates of albumin with angiotensin and ACTH, and albumin treated with "Morpho CDI" alone, gave little or no cross-reaction with antiserum to bradykinin. The occasional persistence of slight complement fixation with the other albumin conjugates may indicate antibodies specific for the *N*-protein urea linkage, a linkage common to both carbodiimides. Another possibility that has not been ruled out is the existence of antibodies to albumin which might have been structurally altered by the action of carbodiimides. Bradykinin itself did not fix complement with the antiserum.

Free bradykinin inhibited the complement-fixing reaction of antiserum to bradykinin with the RSA-bradykinin conjugate (Fig. 3*B*). Neither angiotensin nor ACTH inhibited this reaction.

Figure 4 shows the complement-fixation curve and inhibition data with an antiserum against angiotensin. Specificity of the antigen-antibody reaction is indicated by lack of complement fixation with heterologous hapten-albumin conjugates and lack of inhibition by heterologous haptens.

In comparison with other conjugating procedures, the reaction with carbodiimides has the advantages of occurring under very mild conditions, of conjugating a wide range of compounds, and of conjugating haptens directly to proteins without interposing additional groups between the hapten and the carrier.

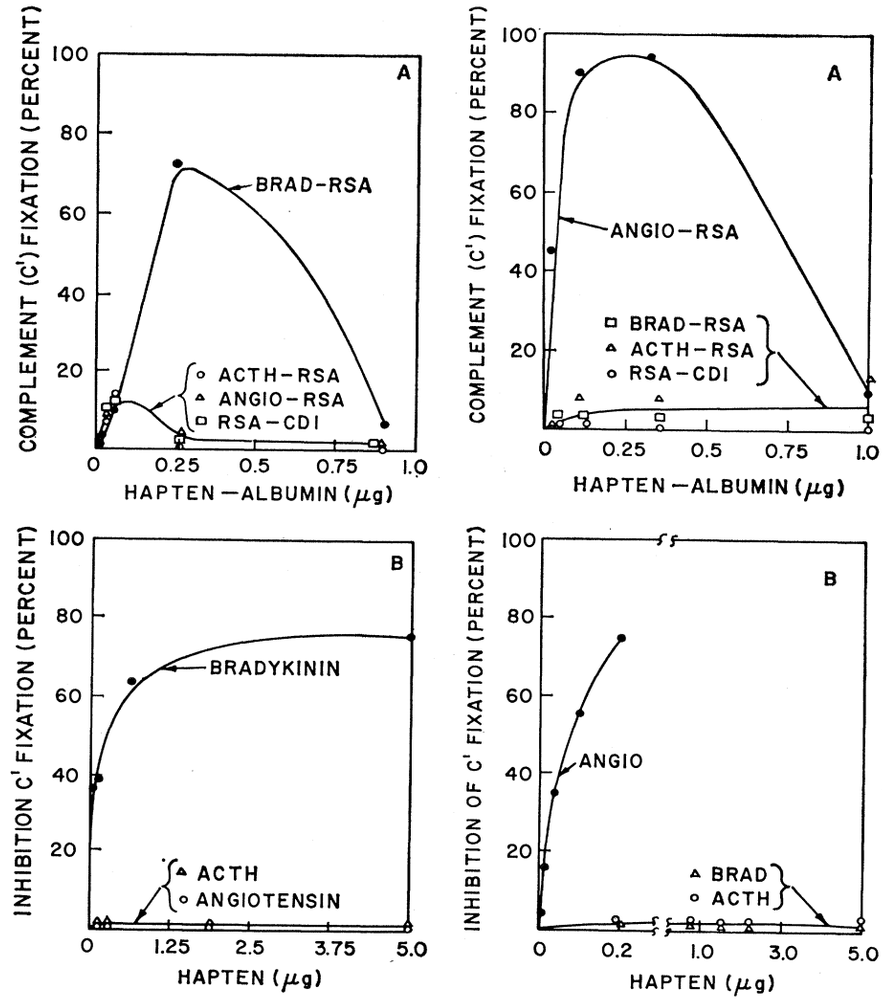

Fig. 3 (left). *A*, Complement fixation by antiserum to bradykinin, diluted 1:2000, and RSA-bradykinin conjugate. The antiserum was prepared by immunizing a rabbit with RSA-bradykinin synthesized with "Ethyl CDI." The antigens used in complement fixation, RSA-bradykinin, RSA-angiotensin, RSA-ACTH, and RSA-carbodiimide were all synthesized with "Morpho CDI." *B*, Inhibition of the complement fixation shown in Fig. 3*A* by bradykinin, angiotensin, and ACTH. The complement fixation was obtained by addition of 0.25 μg of RSA-bradykinin ("Morpho CDI") to the antiserum. *C'* is complement. Fig. 4 (right). Complement-fixation curve with anti-angiotensin, diluted 1:600 (*A*), and inhibition of complement fixation by free haptens (*B*). The antiserum was prepared by immunization with RSA-angiotensin synthesized with "Ethyl CDI"; and the antigens RSA-angiotensin, RSA-bradykinin, RSA-ACTH, and RSA-carbodiimide were synthesized with "Morpho CDI." Inhibition by angiotensin, bradykinin, and ACTH was of a complement-fixation point obtained by addition of 0.25 μg of RSA-angiotensin to the antiserum. *C'* is complement.

We have used the antibodies produced by this method to study the immunochemistry and biological role of small polypeptides (*11*). Future applications of this method may include synthesis of multivalent antigens for detection of antibodies to bradykinin or other haptens or for immunization against toxic compounds of low molecular weight, and for conjugation of antigens to insoluble resins.

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7. Except for our first sample of "Ethyl CDI," which was the gift of Harold Rosenthal, this compound was purchased from Ott Chemical Co., Muskegon, Mich.
8. Purchased from Aldrich Chemical Co., Milwaukee, Wis.
9. Bradykinin was the gift of E. D. Nicolaides, Parke, Davis and Co., and angiotensin was the gift of R. Gaunt, Ciba, Inc.
10. D. H. Spackman, W. H. Stein, S. Moore, *Anal. Chem.* **30**, 1190 (1958).
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13. One rabbit was immunized with a conjugate of albumin and ACTH prepared with carbodiimide. Some of the antibodies produced by this rabbit were directed to ACTH, as shown by ACTH inhibition of complement fixation, and neutralization by the antiserum of the biological activity of ACTH. However, the antiserum also contained a considerable concentration of antibody to carbodiimide derivatives, and unequivocal interpretation of the serological data awaits further study. Porcine ACTH was the gift of A. Cohen, Abbott Laboratories.
14. Valuable assistance was given by Eleanor Wasserman and F. Castillo. Supported by grants from NSF, NIH, and the American Cancer Society, a fellowship from the Helen Hay Whitney Foundation (to T.L.G.), and an established investigatorship of the American Heart Association (to G.D.F.). This is publication No. 277 of the graduate Department of Biochemistry, Brandeis University.

3 February 1964

RNA Synthesis in Rat Seminal Vesicles: Stimulation by Testosterone

Abstract. *Within 70 minutes after the administration of testosterone to rats castrated 12 to 15 hours previously, the rate of synthesis of RNA in the seminal vesicle is increased by 50 percent and continues to rise until approximately 50 minutes after injection when a two- to threefold increase was attained. No further increase was detected for as long as 240 minutes after hormone administration. The base composition of the pulse-labeled RNA was intermediate between that of the total seminal vesicle RNA and DNA-like RNA. No change in this composition was detected at any interval after injection.*

It appears that the synthesis of RNA may be particularly involved in the action of androgenic steroids on accessory reproductive organs (*1*). RNA synthesis in the rat seminal vesicle was increased by testosterone propionate at a time when there was no detectable effect on the synthesis of protein (*1*). This observation suggests that the ultimate growth response of this and other organs on which androgens act may result from an initial enhancement of the synthesis of RNA and perhaps of specific classes of RNA. Liao and Williams-Ashman (*1*) suggested that testosterone governs the synthesis or utilization (or both) of messenger RNA in the ventral prostate. In our study we have determined the time course of the response of RNA synthesis in the seminal vesicle to the administration of testosterone. Incorporation of P^{32} into RNA during a brief interval (pulse labeling) was used as a measure of rate of synthesis, and also permitted analysis of the composition of the newly formed RNA (*2*).

Sexually mature Sprague-Dawley rats (400 to 450 g) were castrated 12 to 15 hours before the intraperitoneal injection of 10 mg of testosterone in saline; injection of saline alone was without effect. Treatment with the hormone took place for various periods of time, while carrier-free P^{32} was administered intraperitoneally 50 minutes before killing. Seminal vesicles were excised and frozen in liquid nitrogen. After the tissues were thawed, they were homogenized in 0.25*M* sucrose and trichloroacetic acid was added to a final concentration of 10 percent. The acid-soluble material was analyzed for inorganic phosphate (*3*) and radioactivity after centrifugation. The insoluble residue was washed several times with trichloroacetic acid and lipid solvents and then extracted with 10 percent NaCl at 100°C to obtain the nucleic acids (*4*). Radioactive contaminants were removed from the nucleic acid extract by the use of diethylaminoethyl cellulose paper in the chloride form (*5*). After hydrolysis of the RNA in 0.5*N* KOH at 37°C for 16 to 18 hours, perchloric acid was added at 0° to 5°C to precipitate any DNA or protein present. Radioactivity and optical density at 260 mμ of the clear supernatant fraction were determined. The specific radioactivity (counts per minute per milligram) of RNA calculated from these determinations was corrected for variations in the specific radioactivity of the acid-soluble phosphate pool. The specific radioactivity of the acid-labile phosphate fraction (obtained by adsorption onto charcoal and heating at 100°C for 7 minutes in 1*N* HCl) corresponded closely with that of the total acid-soluble phosphate fraction. The ribonucleotides in the remaining supernatant

Table 1. Effect of testosterone on composition of total and pulse-labeled seminal vesicle RNA. The composition of total RNA was determined by ultraviolet absorption of eluted nucleotides, while that of newly formed RNA was determined by liquid scintillation counting. Variation in observed values was less than 10 percent.

Time after testosterone (min)	Percentage*				Ratio: (AMP + UMP) / (GMP + CMP)	No. observations
	AMP†	GMP	UMP	CMP		
			Total RNA			
	19	33	20	28	0.65	(12)
			P^{32} *RNA*			
0	22	24	29	26	1.02	(6)
50	23	24	30	23	1.13	(2)
100	23	23	29	25	1.10	(2)
240	24	24	28	25	1.06	(2)
			DNA (ref. 9)			
	28	20	29(T)	23	1.35	

* Percentage of total micrograms of nucleotides or total counts. † AMP refers to adenosine-2′,3′ monophosphate. GMP, UMP, and CMP refer to the 2′,3′ monophosphates of guanosine, uridine, and cytidine.