for 5 minutes. With either enzyme, all vasopressor activity was lost during a 2-hour incubation. Samples incubated without trypsin or chymotrypsin retained full activity.

Formation of the polypeptide similar to angiotensin I occurred only in incubated reaction mixtures of adrenal extract and renin substrate. No vasopressor product was found in incubated mixtures that omitted either adrenal extract or renin substrate. Similarly, unincubated mixtures containing adrenal extract and renin substrate did not contain a vasopressor compound.

In eight other experiments, the renin-like activity of paired adrenal glands was compared with that of kidneys and whole blood. After a rabbit was killed, the thorax and abdomen were opened, and blood (3 to 4 ml) was taken by puncture of the left ventricle. The syringe contained heparin to give a final concentration no greater than 20 units per milliliter of blood. Then the adrenal glands and kidneys were removed. The dialysis and extraction procedures were as described, except that whole blood was not homogenized or diluted with water. The erythrocytes were hemolyzed by freezing and thawing. The incubation mixtures were scaled down proportionately, so that 0.5-ml samples of tissue extracts could be used. Incubation reactions were stopped after 15 minutes (kidney) to 60 hours (whole blood) by heating in a boiling water bath for 5 minutes. The clear supernatants collected after centrifugation were assayed for angiotensin. With an initial renin substrate concentration of 1000 ng of angiotensin content per milliliter, adrenal extracts released angiotensin at rates of 13 to 54 ng per hour per gram (wet weight) of original tissue, that is, at rates about 10 to 40 times greater than that of whole blood. Kidney extracts were 250 to 800 times more active than adrenal extracts.

These data show that the rabbit adrenal gland contains an enzyme that is like renin in the following respects. The enzyme is nondialyzable, is stable for several hours at *p*H 2.6, 4°C, and does not have an absolute requirement for those metals chelated by EDTA and BAL (*13, 18*). It reacts with renin substrate to form a product capable of causing a quick, transient rise of mean arterial blood pressure. This product is soluble in dilute salt solutions, trichloroacetic acid, and *n*-butanol, but not in diethyl ether. It is not inactivated in a boiling water bath but is inactivated by trypsin and chymotrypsin. On countercurrent distribution, the product behaves like angiotensin I and very unlike either known form of angiotensin II. The activity of the renin-like enzyme in adrenal glands was 10 to 40 times that of whole blood and could not be accounted for by trapped blood or plasma.

Whether this enzyme has access to and reacts with renin substrate in vivo remains to be determined. However, in view of the profound effects of angiotensin on aldosterone and catecholamine secretion, it is possible that the adrenal enzyme, by releasing angiotensin, influences adrenal secretions.

JAMES W. RYAN*
Department of the Regius Professor of Medicine, University of Oxford, Oxford, England

References and Notes

1. E. Werle, K. Baumeister, A. Schmal, *Arch. Exp. Pathol. Pharmakol.* **244**, 21 (1962); G. Stakemann, *Acta Pathol. Microbiol. Scand.* **50**, 350 (1960); F. Gross, G. Schaechtelin, M. Ziegler, M. Berger, *Lancet* **1964-I**, 914 (1964); A. B. Gould, L. T. Skeggs, J. R. Kahn, *J. Exp. Med.* **119**, 389 (1964).
2. J. W. Ryan and M. R. Lee, in *Abstracts*, Third International Pharmacological Congress, São Paulo (1966), p. 274.
3. S. M. Hilton and G. P. Lewis, *Brit. Med. Bull.* **13**, 189 (1957).
4. N. Goormaghtigh and H. Handovsky, *Arch. Pathol.* **26**, 1144 (1938).
5. E. Granzer, *Naturwissenschaften* **39**, 405 (1952).
6. W. Feldberg and G. P. Lewis, *J. Physiol.* **171**, 98 (1964); R. L. Robinson, *J. Pharmacol. Exp. Therap.* **156**, 252 (1967).
7. J. H. Laragh, M. Angers, W. G. Kelly, S. Lieberman, *J. Amer. Med. Ass.* **174**, 234 (1960); P. J. Mulrow and W. F. Ganong, *Yale J. Biol. Med.* **33**, 386 (1961); C. C. J. Carpenter, J. O. Davis, C. R. Ayers, A. Casper, *J. Clin. Invest.* **40**, 2026 (1961).
8. R. M. C. E. Dawson, C. Daphne, W. H. Elliott, K. M. Jones, Eds., *Data for Biochemical Research* (Oxford Univ. Press, Oxford, England, 1959), p. 196.
9. Soybean trypsin inhibitor was obtained from Sigma Chemical Co., St. Louis, Missouri.
10. The BAL in arachis oil was obtained from Boots Pure Drug Co., Ltd., Nottingham, England.
11. J. W. Ryan and T. F. Ferris, *Biochem. J.* **105**, 16C (1967).
12. Chlorhexidine gluconate was obtained from Imperial Chemical Industries, Wilmslow, Cheshire, England.
13. J. W. Ryan, J. K. McKenzie, M. R. Lee, in *Abstracts*, Third International Congress of Nephrology, Washington, D.C. (1966), p. 265.
14. L. T. Skeggs, W. H. Marsh, J. R. Kahn, N. P. Shumway, *J. Exp. Med.* **99**, 275 (1954).
15. W. S. Peart, A. M. Lloyd, G. N. Thatcher, N. Payne, N. Stone, A. F. Lever, *Biochem. J.* **99**, 708 (1966).
16. 5-Isoleucine-angiotensin I was the gift of Dr. L. T. Skeggs, Cleveland Veterans Hospital, Cleveland, Ohio; 5-valine-angiotensin II-β-amide was from Ciba, Basel, Switzerland.
17. Trypsin and chymotrypsin preparations were the Tryptar and Chymar products of Armour Pharmaceutical Co., Ltd., Eastbourne, England.
18. E. Braun-Menéndez, J. C. Fasciolo, L. F. Leloir, J. M. Muñoz, A. C. Taquini, in *Renal Hypertension* (Thomas, Springfield, Ill., 1946).
19. I thank Sir George W. Pickering for encouragement and advice, and C. Hanson for technical assistance and art work.
* Present address: The Rockefeller University, New York 10021.

8 November 1967

Efficiency of the First Component of Complement (C'1) in the Hemolytic Reaction

Abstract. *With the use of the first component of guinea pig complement (C'1) labeled in vivo with ^{14}C-amino acids, we have obtained evidence that, under the conditions required for the assay of C'1, each molecule of C'1 capable of interaction with cell surface antigen-antibody complexes is capable of initiating the reaction sequence that leads to lysis of the cell.*

The lysis of cells by antibody and complement (C') results from the sequential interaction of cell-surface antigen, corresponding antibody, and the nine recognized components of C' (*1*). Analysis, on a molecular basis, of the interaction of the complement components has been possible since the development of modern tools of molecular biology and the introduction of the single-site (one-hit) theory of immune hemolysis.

The single-site theory of immune hemolysis states that the completion of the complement reaction-sequence at a discrete site on a cell surface is necessary and sufficient for lysis of the cell (*2*). This theory, however, does not exclude the possibilities that hemolytically inactive C' components may participate in the reaction sequence at a site that does not lead to lysis nor that more than one molecule of any of the C' components may act at a single site. Molecular analyses of the hemolytic dose-response curves for C'1a (the activated form of the first component of C') of guinea pig and human complement indicated that a single effective molecule of this component is sufficient to initiate the reaction-sequence leading to lysis of the cell (*3*). Because these measurements were based solely on hemolysis, however, it was not possible to determine whether every molecule of C'1a was hemolytically active.

Becker (*4*) has shown C'1a to be an enzyme whose substrates are C'4 and C'2 (*4, 5*). Reutilization of C'1a due to transfer from site to site at the cell surface may lead to an overestimation of hemolytic C'1a. Hemolytic C'1a is assayed under conditions where transfer of C'1a from site to site at the cell surface is about 1 percent (*3*), and therefore it cannot be reutilized to any significant degree.

In the assay of C'1a one could underestimate C'1a content owing to in-

efficient conversion of SAC′1a,4,2a to a lytic site (*6*). It has been shown that under the conditions for the assay of C′2 the efficiency of conversion of SAC′1a,4,2a to a lytic site by C′EDTA (*7*) is about 70 percent (*8*). The number of hemolytically effective C′1a molecules is calculated by correcting for the efficiency of conversion of SAC′1a,4,2a to a lytic site.

Recently evidence was reported that, under conditions for the assay of C′2 activity, some SAC′4 sites that were reactive with C′2 could not be converted to a lytic site (*9*). In the same report it was also shown that the hemolytic assay of C′1a activity is not affected by the presence of such sites on EAC′4 (*10*).

In this report evidence is presented that, under the conditions required for the assay of C′1a (*3*), each molecule of guinea pig C′1a capable of interaction with cell surface antigen-antibody complexes is also capable of generating a hemolytic site.

Each of five Hartley guinea pigs was injected with a yeast protein hydrolysate-^{14}C (0.5 mc/kg of body weight) (*11*). The guinea pigs were injected either intravenously or intraperitoneally and were bled by heart puncture 3 to 3½ hours after the injection. Preliminary investigation showed maximum incorporation of label into C′1a protein 3 hours after injection of the ^{14}C-protein hydrolysate.

Serums from these guinea pigs were pooled and a euglobulin precipitate of the serum pool was prepared by the method described by Borsos and Rapp (*3*). The precipitate was resuspended in 0.15*M* NaCl and a 1.2-ml portion was divided into three aliquots, each of which was centrifuged in a 10 to 30 percent sucrose density gradient (ionic strength = 0.065) at 25,000 rev/min for 17 hours (SW 40 rotor, Spinco model L ultracentrifuge). Fractions containing high C′1a activity were pooled. The pool contained 274 μg of protein per milliliter (*12*) and 1.60×10^{13} hemolytically effective molecules of C′1a per milliliter (*13*).

Portions of a 1/10 dilution of this preparation of partially purified, ^{14}C-labeled C′1a were mixed with sheep erythrocytes (E) or with sheep erythrocytes sensitized with Forssman antibody (EA) (*14*). After a 20-minute incubation at room temperature, the cells were separated from the supernatant fluid by centrifugation. The uptake of C′1a was estimated by measurement of the cell-bound C′1a by the C′1a fixation and transfer test (*15*) and by determination of C′1a remaining in the fluid phase by titration of C′1a activity. Portions of these cell preparations and supernatant fluids were assayed for ^{14}C content in the following way. One-tenth-milliliter aliquots of the supernatant fluids were dissolved in 1 ml of NCS reagent (obtained from Nuclear-Chicago Corp.). The cell preparations were washed four times in veronal buffered saline-sucrose solution (*14*) (ionic strength = 0.065) and then lysed in 10 ml of water. The resulting stromas were collected by high-speed centrifugation and the stromas were then dissolved in 1 ml of NCS reagent. Each sample was suspended in 10 ml of scintillation fluid (*16*) and counted for 10 minutes in a Tri-Carb liquid scintillation counter. The results summarized in Table 1 show that under conditions when more than 95 percent of the hemolytic C′1a activity was taken up by EA approximately 10 percent of the ^{14}C was taken up. More than 75 percent of the C′1a fixed by EA was detected on the cells by C′1a fixation and transfer. Under the same conditions E took up negligible quantities of radiolabel and C′1a. Both the radioactivity and C′1a fixed to EA can be completely removed from the cells by 0.01*M* Na–ethylene diamine tetraacetate buffer, *p*H 7.5.

Table 1. Efficiency of ^{14}C-labeled guinea pig C′1a (cpm = counts/min).

Cell concentration (cells/ml)	Effective C′1a molecules (% uptake*)		Radioactivity (cpm/ml)†				Hemolytically effective C′1a		
			Partially purified preparation (initial)	Uptake by		Percent of label on EA	Molecules per milliliter	C′1a/total protein (%)‡	Efficiency§
	EA	E		EA	E				
				Experiment 1					
1×10^9			6460	670	20	10	$1.60 \pm .08 \times 10^{13}$	9.7	0.97
				Experiment 2¶					
1×10^9	97.4	4.1	7520	710	0	8.4	$1.44 \pm .07 \times 10^{13}$	8.7	0.98
2×10^9	98.5	5.3	6740	570	0	9.4			

* Between 76.7 and 84 percent of bound C′1a was detected on the cell surface by C′1a fixation and transfer test. † Duplicate samples differed by less than 10 percent. ‡ Molecular weight of C′1a estimated to be 1,000,000. § Efficiency is the ratio of the percent of the total protein as hemolytically effective C′1a to the percent uptake of radiolabeled protein. ¶ Experiment 2 was performed 1 week after experiment 1. There is a slight loss of hemolytic C′1a activity as well as a reduction of the uptake of radioactivity by EA due to gradual inactivation during storage of the preparation.

Molecular analysis of hemolytic titrations showed that, of the total protein in a fresh preparation of partially purified ^{14}C-labeled C′1a, 9.7 percent was C′1a. Thus, from the data obtained by titration of C′1a activity, by C′1a fixation and transfer, and by uptake of radioactive label, we calculated that approximately 10 percent of the protein in this partially purified preparation is C′1a and that 97 percent of the C′1a identified by radioactivity is hemolytically active. These results were interpreted to mean that under these conditions every molecule of C′1a taken up by EA is hemolytically active. The calculation of C′1a efficiency was based on the assumption that the specific radioactivity of the C′1a in this preparation was not significantly less than that of the contaminating proteins. If the specific radioactivity of the C′1a were greater than that of the other proteins, estimates of C′1a efficiency would be greater than 97 percent.

To test whether the specific radioactivity of the C′1a was significantly different from that of the other proteins, the following experiment was performed. A precipitate of egg albumin and rabbit anti-egg albumin was prepared at equivalence and thoroughly washed with 0.15*M* NaCl. One-milliliter portions of the ^{14}C-labeled C′1a preparation were mixed with a 20-mg or a 2-mg portion of this precipitate. Control mixtures consisted of 20-mg or 2-mg portions of the precipitate and 1-ml portions of the buffer used in preparation of C′1a. All mixtures were incubated for 20 minutes at room temperature and the precipitates were then removed by high-speed centrifugation. The amount of ^{14}C-label, the amount of protein (measured spectrophotometrically), and the number of effective molecules of C′1a remaining in the supernatant fluids were determined. Twenty milligrams of the precipitate removed 54.5 percent of the protein, 59 percent of the ^{14}C-label, and more than 99 percent of the hemolytic C′1a from the partially purified C′1a preparation. Two milligrams of the precipitate removed 32 percent of the protein, 34.2 percent of the ^{14}C-label, and 99.9 percent of the hemo-

lytic C'1a. As stated previously, we found that approximately 10 percent of the ^{14}C was taken up specifically by EA. Since nearly all detectable hemolytically active C'1a was taken up by 20 mg of precipitate as well as by 2 mg of precipitate, it can be concluded that 17 percent of the radiolabel taken up by 20 mg of precipitate was C'1a and that 29 percent of the radiolabel taken up by 2 mg of precipitate was C'1a. Thus, increasing amounts of immune precipitate took up increasing amounts of protein and label in nearly one to one correspondence, while each level of immune precipitate took up more than 99 percent of the hemolytic C'1a, that is the ratio of C'1a to total protein varied while the ratio of protein to radioactivity remained constant (*17*). These results strongly support the assumption that the specific radioactivity of C'1a is similar to that of other proteins in the partially purified preparation of C'1a. An error of 30 percent in the estimate of specific radioactivity of C'1a would be detected by these methods. Therefore, we concluded that less than 1.4 molecules of C'1a is required to generate a single hemolytic site.

Estimates of the hemolytic efficiency of other complement components have been made with ^{125}I-labeled human C'4 and C'3 (*18*). According to these studies, uptake of 15 to 40 C'4 molecules and several hundred C'3 molecules accompanied the generation of a single effective hemolytic site.

HARVEY R. COLTEN
TIBOR BORSOS
HERBERT J. RAPP
Biology Branch, National Cancer Institute, Bethesda, Maryland 20014

References and Notes

1. R. A. Nelson, *The Inflammatory Process* (Academic Press, New York, 1965), p. 819.
2. E. A. Kabat and M. M. Mayer, *Kabat and Mayer's Experimental Immunochemistry* (Thomas, Springfield, Ill., ed. 2, 1961), chap. 4.
3. T. Borsos and H. J. Rapp, *J. Immunol.* **91**, 851 (1963).
4. E. L. Becker, *ibid.* **84**, 299 (1960).
5. R. M. Stroud, K. F. Austen, M. M. Mayer, *Immunochemistry* **2**, 219 (1965).
6. SA, site sensitized with antibody.
7. This is 1 : 50 guinea pig serum in 0.01*M* ethylenediaminetetraacetate.
8. T. Borsos, H. J. Rapp, M. M. Mayer, *J. Immunol.* **87**, 310 (1961).
9. ———, *ibid.* **99**, 213 (1967).
10. EA, sheep erythrocytes sensitized with Forssman antibody.
11. Schwarz BioResearch lot No. 6602, specific activity 1500 μc/mg.
12. Determined spectrophotometrically. A comparison of absorption at 280 mμ (light path 1 cm) and determination of nitrogen by nesslerization on partially purified C'1a preparations (approximately 60 percent of protein was active C'1a) indicated that 1.0 optical density unit = 1.03 mg of protein.
13. Estimates of the number of molecules of C'1a by hemolytic activity were slightly less when measured at a later time (see Table 1).
14. The diluent used in these experiments was veronal buffered saline sucrose at an ionic strength of 0.065 with Ca^{++} 0.00015*M*, Mg^{++} 0.0001*M* and 0.1 percent gelatin. Antibody supplied in quantity sufficient to generate 5×10^{12} C'1a fixing sites per milliliter.
15. T. Borsos and H. J. Rapp, *J. Immunol.* **95**, 559 (1965).
16. Solution of 5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenoxazolyl)] benzene in 1 liter of toluene.
17. The nature of the radioactive protein other than C'1a taken up by specific immune precipitates is not known. It may represent nonspecific trapping or specific uptake of the protein. At present we cannot distinguish between these possibilities; however, the mechanism of uptake and nature of the protein do not affect the conclusion that the specific radioactivity of C'1a is similar to that of the other proteins in the partially purified C'1a preparations.
18. N. Cooper and H. J. Muller-Eberhard, in *Proceedings of the XV Colloquium: Protides of the Biological Fluids* (Elsevier, Amsterdam, in press); H. J. Muller-Eberhard, A. P. Dalmasso, M. A. Calcott, *J. Exp. Med.* **123**, 33 (1966).
19. We thank Dr. R. A. Lazzarini for his assistance and helpful advice.

5 October 1967

Olfactory Input to the Hypothalamus: Electrophysiological Evidence

Abstract. *Electrical stimulation of the rat's olfactory bulb or lateral olfactory tract elicited unit discharges in the region of the medial forebrain bundle of the lateral hypothalamus, with latencies of 4 to 25 milliseconds. Unit responses in this area were driven by odors in preparations that were paralyzed to prevent breathing artifacts.*

Anatomic reports point strongly to olfactory influences on the hypothalamus through the medial forebrain bundle (*1*); these connections are probably important in some of the behavioral and endocrinologic functions of the hypothalamus. There is good evidence of hypothalamic function in feeding and in sexual behavior, and it is generally believed that olfaction plays a significant role in guiding these behaviors. The role of olfaction in sex is now receiving particular attention. Male rats prefer the odor of estrous females to nonestrous (*2*), and male rats having their olfactory bulbs removed show reduced ability to mate (*3*). Odors have potent effects also on pregnancy and on endocrine functions in mice (*4*). Experimental evidence of olfactory control of feeding is less complete, but it is known that olfaction can be the basis of aversions to foods (*5*).

One electrophysiologic study of the response of hypothalamic cells to many stimuli showed that a significant portion changed their activity when odors were presented (*6*). As a preliminary step in investigation of the importance of the olfactory hypothalamic connections, our study was planned to extend the observation of olfactory responses in the hypothalamus, particularly in the lateral area, and to ascertain the distribution of neurons responsive to olfaction.

In the initial experiments we searched the lateral hypothalamus for responses to electrical stimulation of the olfactory bulb or the lateral olfactory tract in 13 Sprague-Dawley albino rats. The anesthesia was a mixture of 75 mg of chloralose and 1.5 mg of urethane, per kilogram of body weight, dissolved in polyethylene glycol–200. Bipolar electrodes and differential amplification were used for recording. The electrodes were constructed of 60-μ nichrome wire, with the tips separated by about 0.2 mm. Square-wave electrical stimulation of 0.1- to 1.0-msec duration and up to 15 volts was delivered with bipolar electrodes either to the olfactory bulb or to the lateral olfactory tract on the same side as the recording electrode. The olfactory bulb was approached dorsally by thinning the overlying skull with a dental burr and removing the remaining bone and meninges with fine forceps. The bipolar stimulation electrode, bared for about 1 mm back from the tip, was then inserted into the bulb. The lateral olfactory tract was exposed in the same manner from the base of the orbit, and the electrode was placed on the tract, under visual control.

Our search centered mainly on the portion of the lateral hypothalamus from the optic chiasma to the mammillary nuclei; it was bounded medially by the fornix and laterally by the internal capsule. Seventy-five penetrations were made in this region with the bipolar recording electrodes. When a responsive area was found we generally halted penetration of the electrode and made a small lesion by passing current between the two electrode tips to mark the responsive areas for histologic identification. After each experiment the rat was perfused with Ringer solution and 10 percent formalin. Alternate frozen sections 50 μ thick were cut and stained with Weil and cresyl violet.

In the lateral ventral portion of the medial forebrain bundle we often found units or groups of units that responded to the electrical stimulus, with latencies ranging from 4 to about 25 msec. Many units that could be driven by electrical stimulation could also be activated with odors. Stimulation con-