represented a difference between choline influx and sodium outflux it was obvious that choline movement, if any, must be small and probably passive.

Thus, Koblick's contention that skins of *R. pipiens* are not impermeable to choline has been confirmed. The reasons for the discrepancy between our earlier work and these experiments are unknown. Possibly a species difference is involved, since skins of *R. esculenta* were used in the older experiments. On the other hand, the magnitude of the choline flux is very small, and for most purposes the ion can be considered to be virtually nonpenetrating, especially if skins offering average resistance to diffusion are used. There is no evidence for an active choline transport across the skins tested (*R. esculenta* and *R. pipiens*) (*6*).

LEONARD B. KIRSCHNER
Department of Zoology, Washington State University, Pullman

References and Notes

1. L. B. Kirschner, *J. Cellular Comp. Physiol.* **45**, 61 (1955).
2. K. Zerahn, *Acta Physiol. Scand.* **33**, 347 (1955).
3. D. C. Koblick, *J. Gen. Physiol.* **42**, 635 (1959).
4. F. Morel, *La Méthode des Indicateurs Nucleaires dans l'Etude des Transports Actifs d'Ions* (Pergamon, Paris, 1959), pp. 155–184.
5. H. H. Ussing and K. Zerahn, *Acta Physiol. Scand.* **23**, 110 (1951).
6. This work was supported by funds provided for biological and medical research by the State of Washington Initiative Measure No. 171, and by a grant-in-aid (G-4925) from the National Science Foundation.

14 March 1960

Isolation of Antibodies to Gelatin from Antigen-Antibody Complex by Proteolysis

Abstract. Antibodies were isolated by digestion of the gelatin-antigelatin complex with collagenase and removal of the gelatin fragments by dialysis. The pure antibody could be precipitated anew by the antigen. Antibodies to tyrosylgelatin were isolated in a similar way, but in this case peptides containing the active antigenic sites remained bound to the antibody.

Antibody preparations of high purity are a prerequisite for the study of the mechanism of antibody formation and mode of action. The purification of antibodies is based either on physicochemical procedures or on immunospecific reactions. By the second method antibodies have been purified with the help of insoluble antigens, as well as by the elution of antibody from antigen-antibody complexes by heat, strong salt solutions, alkali, or acids (*1*). Antibodies have also been purified by precipitation with slightly modified antigens, followed by dissolution in alkali or acid and selective precipitation of the modified antigen (*2*, *3*).

Enzymatic cleavage of the antigen in the antigen-antibody complex would be a very convenient method for the isolation of antibodies, if such treatment would not affect the antibody molecules. In the case of diphtheria and tetanus toxin-antitoxin floccules, digestion with pepsin or trypsin yielded preparations of antitoxins of high activity, but their molecular weights were reduced to about half the original size (*1*). In this report (*4*) we describe the isolation of pure, undamaged antibodies to gelatin by selective digestion of the antigen in the gelatin-antigelatin precipitate by collagenase. This enzyme degrades collagen and gelatin to products of an average molecular weight around 500, but seems to be inactive against other proteins (*5*).

In a control experiment, gelatin (U.S.P. granular, Fisher) was digested with collagenase (*6*) (4 units of collagenase per milligram of gelatin) in 0.05*M* tris buffer, *p*H 7.4, containing 0.005*M* $CaCl_2$, at 25°C, for 3 hours. The reaction mixture was subjected to exhaustive dialysis against water at 2°C for 3 days, and the solution both inside and outside the dialysis bag were hydrolyzed (6*N* HCl, 110°C, 24 hr). All the hydroxyproline of the original gelatin was found (*7*) on the outside, while none remained in the dialysis bag. This shows that the gelatin was split quantitatively into dialyzable fragments. When human γ-globulin was treated with collagenase under similar conditions but in phosphate buffer, no dialyzable peptides were split off (Kjeldahl nitrogen analysis). It appeared, in fact, that the γ-globulin molecules were completely unchanged by the enzyme, as the material sedimented at the same rate before and after treatment with collagenase in tris buffer ($S_{20} = 6.6$, at a 0.17 percent concentration in 0.15*M* NaCl; Spinco model E ultracentrifuge, at 56,100 rev/min), and the area enclosed by the gaussian curve in the sedimentation pattern remained constant.

Rabbit antiserum to gelatin was prepared and the antibodies were precipitated with gelatin, according to Maurer (*8*). The precipitate resulting from the addition of 0.65 mg of gelatin to 13 ml of antiserum was washed repeatedly with 0.15*M* NaCl. It contained 0.25 mg of antigen (33 μg of hydroxyproline in the hydrolyzate) and 1.53 mg of antibody (derived from the extinction at 280 mμ of the neutralized solution of the precipitate in 0.1*N* NaOH). A suspension of this precipitate in 2 ml of 0.05*M* tris buffer, *p*H 7.4, containing 0.005*M* $CaCl_2$ was treated with collagenase (3 units), at 25°C. After 3 hours the remaining insoluble material

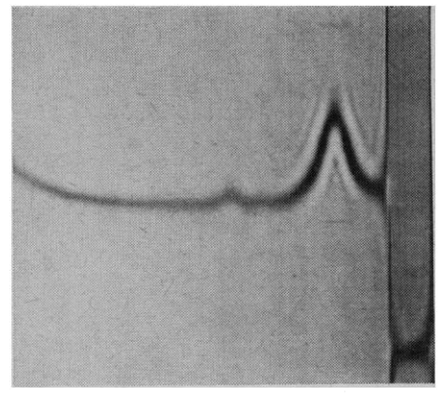

Fig. 1. A sedimentation pattern, in a Spinco model E ultracentrifuge, of antityrosylgelatin (0.17 percent in 0.15*M* NaCl), at 20°C. The photograph was taken 16 minutes after full speed (56,100 rev/min) was attained.

was centrifuged off. It contained 26 percent of the gelatin in the original precipitate (65 μg of gelatin, as calculated from hydroxyproline data). The supernatant fluid was subjected to exhaustive dialysis against the buffer, at 2°C. After 3 days the contents of the dialysis bag were brought up to 3.5 ml. The fact that hydroxyproline was found in the hydrolyzate of this solution, demonstrated that no gelatin-split products were present. The solution contained 80 percent of the antibody in the original precipitate (0.35 mg of antibody per milliliter, as calculated from the extinction at 280 mμ). Only one spot was detected on paper electrophoresis, migrating identically with the γ-globulin in rabbit normal serum. The material showed a sedimentation constant of S_{20} equal to 7.14 in the ultracentrifuge [rabbit γ-globulin has a sedimentation constant S_{20} approximately equal to 7 (*1*)]. The purified antibody could be precipitated (*8*) anew by the addition of gelatin.

Gelatin is a weak antigen; it yields antisera of low antibody titer, and the precipitin reaction takes a prolonged time. Attachment of tyrosine peptides to gelatin converts it into a relatively powerful antigen (*9*). In view of this enhancement of antigenicity, the possibility of obtaining pure antibodies from the system polytyrosylgelatin-antipolytyrosylgelatin was also investigated. A gelatin derivative enriched with only 2 percent tyrosine (*10*) ("tyrosylgelatin") served as antigen in this experiment. It had been shown previously (*9*) to be a strong antigen in comparison to gelatin. Upon digestion with collagenase and exhaustive dialysis (conditions similar to the control experiment with gelatin) tyrosylgelatin was split into fragments that dialyzed out completely, as followed spectrophotometrically (293.5 mμ, *p*H 13), as

well as by the hydroxyproline analysis of the hydrolyzates (*7*).

The precipitate obtained by the addition of 3 mg of tyrosylgelatin to 25 ml of homologous rabbit antiserum (*9*) (the washed precipitate contained 1.9 mg of antigen and 8 mg of antibody) was treated with collagenase (15 units) as in the case of the gelatin-antigelatin floccules. The suspension became almost completely clear during the enzymic reaction. The fraction that dialyzed out accounted for 90 percent of the hydroxyproline present in the precipitate. The dialysis bag contained 85 percent of the antibody in the original precipitate (as calculated from the extinction at 280 mμ), as well as the remaining 10 percent of hydroxyproline. When subjected to paper electrophoresis, the antibody solution showed one spot only, with the mobility of normal rabbit serum γ-globulin. On sedimentation (Fig. 1) the solution was shown to contain a principal component with S_{20} equal to 7.06 and small amounts (approximately 6 percent) of a component with S_{20} equal to 19.2 [a 19 *S* component has been reported to be present in small amounts in many γ-globulins (*1*)]. Upon addition of new tyrosylgelatin to the antibody solution there was almost no precipitation, even when care was taken to inhibit any collagenase activity still present by the addition of Versene (*5*).

In the absence of the respective antibodies, collagenase converts both gelatin and tyrosylgelatin quantitatively into dialyzable fragments. Nevertheless, the possibility could be envisaged where the digestion of the antigen-antibody complex might leave the active site of the antigen bound to the antibody. With gelatin this does not seem to be the case, as the isolated antibody contained no hydroxyproline and could be precipitated with gelatin. On the other hand, in the case of tyrosylgelatin, the purified antibody still contained some hydroxyproline (it would correspond to 3 percent gelatin impurity, if calculated on the base of the composition of the whole antigen molecule) and could not be precipitated by the homologous antigen. This is consistent with the assumption that peptides containing the active site of the antigen are still bound to the antibody molecules. The above argument is further supported by the results of inhibition experiments of the specific precipitin reaction by means of collagenase digests of the antigens. While the collagenase digest of gelatin does not inhibit the precipitation of antigelatin by gelatin (*11*), it was found that the collagenase digest of tyrosylgelatin (the solution that dialyzed out from a mixture of 2 mg of tyrosylgelatin and 6 units of collagenase, after 3 hours of incubation at 25°C) completely inhibited the precipitation of antityrosylgelatin from 0.5 ml of antiserum, by the homologous antigen. It may be thus concluded that the hydroxyproline in the purified antibody solution is derived from fragments of the antigen bound to the active sites of the antibody molecules.

The method used here for the antibody purification is limited to systems where an enzyme may digest the antigen (protein or polysaccharide) in the antigen-antibody complex without damaging the antibody molecules. The active site of the antigen may under these circumstances remain, in some cases, bound to the antibody. This might permit the isolation of the active fragment (*12*).

RUTH ARNON
MICHAEL SELA
Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel

References and Notes

1. H. C. Isliker, *Advances in Protein Chem.* **12**, 387 (1957).
2. L. A. Sternberger and D. Pressman, *J. Immunol.* **65**, 65 (1950).
3. S. J. Singer, J. E. Fothergill, J. R. Shainoff, *J. Am. Chem. Soc.* **81**, 2277 (1959).
4. This paper is part 4 in the series "Studies on the Chemical Basis of the Antigenicity of Proteins"; part 3, by M. Sela and R. Arnon, *Biochem. J.*, in press.
5. S. Seifter, P. M. Gallop, L. Klein, E. Meilman, *J. Biol. Chem.* **234**, 285 (1959); S. Michaels, P. M. Gallop, S. Seifter, E. Meilman, *Biochim. et Biophys. Acta* **29**, 450 (1958).
6. The collagenase (95 units per 1 mg) was kindly provided by Dr. P. M. Gallop.
7. R. E. Neuman and M. A. Logan, *J. Biol. Chem.* **184**, 299 (1950).
8. P. H. Maurer, *J. Exptl. Med.* **100**, 497, 515 (1954).
9. M. Sela and R. Arnon, *Biochem. J.*, **75**, 91 (1960); R. Arnon and M. Sela, *ibid.* **75**, 103 (1960).
10. In this derivative, on the average, single units of L-tyrosine are attached by peptide linkage to half of the amino groups of gelatin.
11. P. H. Maurer, *J. Exptl. Med.* **107**, 125 (1958).
12. This work was supported by grant A-3083 of the National Institutes of Health, U.S. Public Health Service.

8 January 1960

Electronic Structure and Nicotine-like Stimulant Activity in Choline Phenyl Ethers

Abstract. Frontier electron density at the ether oxygen position and superdelocalizability at the ortho position show good parallelism with biological activity—that is, the stimulant activity of phenyl ether choline molecules. The mechanism of the biological action is discussed in connection with this finding.

The nicotine-like stimulant activity of phenyl ether cholines varies greatly with the substituents (*1*), but the mechanism of this activity remains obscure. The idea that the electron density at some points in the molecule, rather than the presence of some particular group, is important for the pharmacological activity, has been recognized. However, no quantum-mechanical approach in understanding the mechanism has been made.

We have established the frontier electron theory as one of the quantum-mechanical theories of organic chemical reaction, and its prediction agrees with experimental results better than previous theories (*2–4*). Furthermore, the theory has been successfully applied to the problem of some biological actions of conjugated molecules, such as the carcinogenic activity of polycondensed aromatic hydrocarbons (*5*) and the plant-growth activity of benzoic acid derivatives (*6*).

In this report, the frontier electron theory is further extended to explain the nicotine-like stimulant activity of phenyl ethers of choline. The theoretical indices used in this report are (i) frontier electron density (*2*) and (ii) superdelocalizability (*3*). These were derived as the reactivity indices of two extreme cases of stabilization at the transition state, due to the charge transfer from the substrate molecule to the pseudo-π orbital (that is, a π-like orbital which, according to the theory, comes into being near to the transition state, and consists of the orbitals in the reagent and the atom to be attacked in the substrate molecule), or vice versa. Let α be the coulomb integral of a carbon atom in benzene, and let h be the energy of the pseudo-π orbital. Then the frontier electron density and the superdelocalizability correspond to the case in which h is equal to the frontier orbital energy and to the case in which h is equal to α, respectively. The larger these indices are, the more reactive the position in question is, not only in substitution or addition reaction, but also in molecular complex formation.

Superdelocalizability has the following explicit formulas according to the type of reaction:
electrophilic reaction:

$$S_r^{(E)} = 2 \sum_j^{\text{occ}} (C_r^j)^2/\lambda_j$$

radical reaction:

$$S_r^{(R)} = \sum_j^{\text{occ}} (C_r^j)^2/\lambda_j + \sum_j^{\text{unocc}} (C_r^j)^2/(-\lambda_j)$$

nucleophilic reaction:

$$S_r^{(N)} = 2 \sum_j^{\text{unocc}} (C_r^j)^2/(-\lambda_j)$$

where C_r^j is the coefficient of the rth atomic π orbital in the jth molecular orbital, λ_j is the coefficient in the equation $\epsilon_j = \alpha + \lambda_j \beta$, ϵ_j is the energy of the jth molecular orbital, and α and β are the coulomb and resonance in-