study the *in vitro* digestibility of this protein in comparison with raw (undried) egg white and coagulated egg white.

The existence of an anti-tryptic principle in raw, undried egg white has been conclusively demonstrated[3] and our first experiment was designed to show whether this principle existed in the commercially dried product. Thereafter, comparison of the course of peptic digestion of the dried and undried egg white with that of coagulated egg white was undertaken. Experiments by Frank[4] have shown that the course of peptic digestion is affected by the conditions of coagulation. In the experiments to be described attempts were made to hold this factor constant in successive trials.

All tests were carried out at 37° ± 1° C. using a commercial sample of dried egg white[5] and locally purchased fresh eggs as the source of undried and coagulated egg white. Enzymes were regular commercial samples of hog pancreatin and pepsin, each studied at its respective pH optimum. Criteria of digestion were, for pancreatic digestion, the increase in nitrogen not precipitable in 7.5 per cent. trichloroacetic acid and, for peptic digestion, the increase in nitrogen not coagulable by heat and acid.

Table 1 shows that commercially dried egg white

TABLE 1

PANCREATIC DIGESTION OF VARIOUS EGG WHITE PREPARATIONS

	Raw egg white		Coagulated egg white
	Undried	Dried	
Total N mg/cc	2.78	2.63	2.61
Ratio, substrate N : enzyme N	60	57	57
Time	% total N soluble in 10 per cent. trichloroacetic acid		
0 hr.	4.0	3.8	4.3
2 "	4.0	4.3	20.4
8 "	4.2	4.4	35.4
24 "	4.8	5.1	43.0

All solutions were adjusted to pH 7.3–7.4 and maintained within that range during the experiment.

contains about as much antitryptic principle as fresh (undried) egg white. The data of Table 2 show that, under the conditions studied so far, no real difference exists between the courses of peptic digestion of dried and undried raw egg white, but both are inferior in digestibility to coagulated egg white.

Further experiments are in progress to establish conditions within the physiologic range necessary to inactivate the anti-tryptic principle in raw egg white.

[3] H. A. Balls and G. L. Swenson, *ibid.*, 106: 409, 1934.
[4] P. Frank, *ibid.*, 9: 463, 1911.
[5] The dried egg white preparation was a Swift product used in dog-feeding experiments conducted by Dr. James B. Allison at Rutgers University. Grateful asknowledgment is made to Dr. Allison for this material.

TABLE 2

PEPTIC DIGESTION OF VARIOUS EGG WHITE PREPARATIONS

	Raw egg white		Coagulated egg white
	Undried	Dried	
Time	% increase in non-heat and acid coagulable N		
1 hr.	61	56	98
2 "	88	90	127
4 "	138	143	195

All mixtures were maintained at pH 1.8. Commercial hog pepsin (1 : 10,000) was used in the approximate ratio of 1 part pepsin N to 1,000 parts substrate N.

Haurowitz *et al.*[6] have very recently suggested the possibility that impaired gastric function (achylia) may prevent utilization of undenatured globular proteins by the animal organism. Their experiments, carried out with crystalline trypsin on native and denatured proteins freed of anti-tryptic principles, have interesting theoretical implications. It is desirable to supplement their studies with experiments, such as those recorded here where conditions more nearly simulate those encountered in physiologic digestion.

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EFFECT OF CONCENTRATED UREA SOLUTION ON THE PRECIPITATING POWER OF ANTIOVALBUMIN: SIGNIFICANCE OF FORMATION OF PROTEIN COMPLEXES

IN order to study the effect of protein denaturing agents on antibodies, it is usually necessary to remove such agents before testing the resulting material for antibody activity. Interpretation of the results must take into consideration, therefore, not only the possible destruction of the intrinsic structure of the antibody molecule, but also secondary effects which occur upon removal of the denaturing agent. There is considerable evidence[1] that aggregation or complexing of protein molecules occurs during certain types of denaturation or upon removal of denaturing agents and that aggregates of various dimensions and solubilities are formed. Those aggregates or complexes which are insoluble except in relatively concentrated dispersing solutions such as strong acids, bases, thiocyanates, etc., are referred to as irreversibly denatured, while the relatively soluble materials are designated either as native (unaffected) or "renatured" protein. However, it is generally true that

[6] F. Haurowitz, M. Tunca, P. Schwerin and V. Goksu, *Jour. Biol. Chem.*, 157: 621, 1945.
[1] Discussed in review by Hans Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Rev.*, 34: 157, 1944.

solutions of the latter type are not identical to solutions of untreated protein. Solutions of these so-called renatured proteins often show properties which clearly indicate that some aggregation has occurred and hence it becomes highly important to consider this possibility in any interpretation of studies dealing with the effect of denaturation on the physical or chemical activity of protein molecules. This is especially true when dealing with such a reaction as that of antigen-antibody precipitation. For example, one might expect that, under certain conditions, complexing of antibody with excess non-antibody protein would result in a non-reactive antibody mixture due merely to the covering of antibody combining sites rather than to any actual destruction of the antibody molecule. Furthermore, under certain conditions the amount of precipitable "antibody protein" might actually increase due to the complexing of antibody and non-antibody protein, but with enough specific combining sites exposed to afford the formation of a framework and subsequent precipitation. Highly purified antibody would show no change attributable to complexing, since there would always be plenty of combining sites exposed. It is possible, however, that under such conditions the destruction of a considerable number of combining sites would be masked by complex formation, since the resulting aggregates would have sufficient combining sites to give precipitation.

Studies on the significance of complexing of denatured antibody systems have been limited almost entirely to the work of Kleczkowski and co-workers,[2] who published a series of papers on the effect of heat denaturation on various antibody preparations. Much of the loss of antibody activity which they obtained was attributed to complexing of antibody protein with serum albumin. Although Erickson and Neurath,[3] in a report on guanidine hydrochloride denaturation of antipneumococcus serum, failed to consider the possibility of complexing, it is of interest that their "regenerated" antibody was almost as active as native antibody and the so-called inversibly denatured antibody showed a greater precipitating power than native antibody tested under similar conditions.

Our own studies on the effect of concentrated urea on the precipitating power of a variety of antibodies leave little doubt that complexing takes place upon removal of urea. This was evident because urea-treated preparations often showed an increase in the amount of precipitable protein at optimum antigen-antibody concentrations, and because the effect was dependent to a significant degree upon the protein treatment with urea. The work reported here deals entirely with studies on rabbit antiovalbumin. A more detailed report dealing with antiovalbumin as well as antibodies against arsaenilic acid, pneumococcus polysaccharide and diphtheria toxin will be published later.

Three types of antiovalbumin preparations were studied, namely, whole antiserum, antibody which had been partially purified by ammonium sulfate precipitation[4] and antibody which had been isolated from a specific precipitate by acid dissociation.[5] All preparations came from a pool of fresh rabbit serum which showed an optimum proportion zone of 1: 6,000 and 2.0 mg of antibody per ml of whole serum. Each type of preparation was treated in three different protein concentrations (see Table 1). Solid urea was added to each sample to give a final concentration of 8 molar. The solution was then adjusted to pH 8.0 and allowed to stand at room temperature for 48 hours. The urea was then removed by dialysis against 1.0 per cent. NaCl solution and the protein concentration was adjusted to a uniform value before making the precipitation tests. The tests were made in the usual manner, using twofold dilutions of antigen, and the resulting precipitates analyzed for nitrogen as described by Pressman.[6]

TABLE 1

THE EFFECT OF UREA ON THE PRECIPITATING POWER OF RABBIT ANTIOVALBUMIN

Type of antibody preparation	Per cent. protein during urea treatment	Ratio of treated to untreated*
Whole serum	5.80	0.00
	1.36	0.89
	0.58	1.05
Partially purified antibody	5.00	1.28
	1.00	1.19
	0.50	1.05
Specifically† purified antibody	2.94	1.01
	0.58	0.98
	0.29	1.02

* Ratio of amount of precipitate obtained in urea-treated sample to amount obtained in untreated sample.
† Approximately 93 per cent. of the untreated total protein precipitated at optimum antigen concentration.

The results obtained are summarized in Table 1. Comparison is made as a ratio of the maximum amount of precipitate obtained with treated preparation with maximum amounts obtained with untreated controls. The sample of undiluted whole serum (5.8 per cent. protein) failed to give a precipitate in any antigen dilution, although there was a zone showing

[2] A. Kleczkowski, *Brit. Jour. Exp. Path.*, 22: 1921, 1941; F. C. Bawden and A. Kleczkowski, *Brit. Jour. Exp. Path.*, 23: 178, 1942; A. Kleczkowski, *Biochem. Jour.*, 37: 30, 1943.

[3] J. O. Erickson and Hans Neurath, SCIENCE, 98: 284, 1943.

[4] Use was made of the fraction obtained at one third saturation with salt. The precipitable antiovalbumin protein was 38 per cent. of the total protein.

[5] Details on the specific purification of various types of antibodies will be given elsewhere. In this particular preparation 93 per cent. of the total protein was precipitable.

[6] David Pressman, *Ind. and Eng. Chem.*, 15: 357, 1943.

faint turbidity in 24 hours. When the concentration of whole serum was reduced to 1.36 per cent. protein during urea treatment, the resulting material showed an activity of 89 per cent. of the activity shown by untreated serum. When the protein concentration was reduced to 0.58 per cent., the activity of the resulting material was approximately the same as the untreated control. The partially purified antibody showed the greatest effect of protein concentration—the 5 per cent. solution giving 28 per cent. more precipitate than the untreated control. Increases of 20 to 40 per cent. have been obtained with most systems of this type or where small amounts of serum albumin were added to purified antibody preparations. The third preparation, which consisted of highly purified antibody, showed essentially no effect from the urea treatment. In no case was there any significant change in the equivalence zone. However, the zones were usually much sharper for the urea-treated preparations.

These results indicate that when there is a large excess of non-antibody protein, optimum conditions for complexing of proteins will tend to eliminate precipitating activity due to masking of combining sites. When there is a relatively large amount of antibody protein present, complexing tends to increase the apparent precipitability of the antibody preparation because of complexing with non-antibody protein but with sufficient combining sites exposed to afford the formation of a precipitating framework. When the antibody is essentially pure, complexing has little or no effect on the resulting preparation. It is apparent, therefore, that considerable care should be exercised in the interpretation of data obtained for denaturation of antibodies, not only with urea but also any denaturing agent which causes the formation of protein complexes.

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SCIENTIFIC APPARATUS AND LABORATORY METHODS

THE TECHNIQUE OF INDUCING SPAWNING IN HALIOTIS RUFESCENS SWAINSON

IN April, 1940, an investigation into the life history of *Haliotis rufescens,* the commercial abalone of California, was initiated at the Hopkins Marine Station of Stanford University. In September of the same year the program was interrupted when the writer accepted temporary employment with the California State Division of Fish and Game. Due to subsequent enlistment in the armed forces, resumption of the work has been indefinitely postponed. It is, therefore, deemed advisable to present the following brief notes on the solution of one difficult problem, lest all results be lost.

Haliotis rufescens is a dioecious gastropod mollusk. Tide-pool and shallow-water observations made in the spring of 1940 revealed that the expulsion of sexual products by the male was of common occurrence, usually easily induced by disturbing the specimen. During any period of desiccation following removal of specimens from their habitat, large quantities of sperm were liberated. Similar treatment, however, never resulted in the liberation of eggs by the females.

Although the difficulty of inducing ovulation in this species has caused the abandonment of at least two previous attempts at an embryological investigation, numerous means were tried in an effort to solve the problem. Methods as drastic as the injection of KCl isotonic with sea water, a technique employed successfully by Dr. Albert Taylor,[1] of the California Institute of Technology on various mollusks, failed. After various methods had been tried with negative results, the following technique was arrived at as a successful method to induce spawning and fertilization.

For each experiment 15 to 20 specimens of both sexes were taken in the littoral waters, varying in depth from four to 18 feet. Since exposure to air was found to be necessary for the success of induced spawning, these animals were brought to the laboratory dry in large tubs. A period of desiccation totalling one hour and fifteen minutes was finally found to represent the optimum time for exposure to air. During this period large quantities of sperm were given off by all male specimens. This sperm was thoroughly washed over the entire body of each female; then all individuals of both sexes were placed in well-aerated salt-water tanks. These concrete tanks were out of doors and simulated quite well the natural environment of the species under discussion. Sperm continued to issue from the males until the water became cloudy and, in successful experiments, spawning of the females occurred within six to eight hours after placing the animals in the tanks.

Fertilization resulted in obtaining of typical mollusk cleavage stages. Development continued until trochophore larvae appeared, each still enclosed in a thin membrane. These membranes were finally ruptured and the trochophores became extremely active free-swimming larvae. Changes continued through the veliger stage, paralleling somewhat the develop-

[1] Personal communication.