Reports

Influence of Sulfhydryl Compounds on Precipitation of Plasma Protein with Ethanol

The use of ethanol to precipitate plasma proteins as the first step in the extraction of free amino acids has disclosed an interesting relationship between the nature of the protein precipitate and the quantity of cysteine in plasma.

When three volumes of 95 percent ethanol are added to plasma from normal individuals, the plasma protein precipitates in a coarse granular form so that the aqueous ethanol supernatant can be rapidly removed by filtration through a sintered glass filter. Plasma from some patients with leukemia, however, has been found to precipitate in such a finely divided state that filtration is difficult. It was thought initially that the striking alteration in the nature of these protein precipitates might be related to changes in the proteins in the patients. This is probably not the case, however, as the following discussion indicates.

It was observed that cysteine was not detectable by paper chromatography in the blood plasma showing this phenomenon. Addition of 2 to 3 μg of cysteine per milliliter of plasma before or after precipitation changed the precipitate to a coarse granular form within a few minutes. Addition of larger amounts of cysteine decreased the time required for conversion of a fine to a coarse precipitate. Low plasma cysteine and altered plasma protein precipitation was observed with blood samples from several patients with chronic lymphocytic leukemia. Patients with acute leukemia and chronic granulocytic leukemia did not show such marked changes from normal. After treatment, marked alterations were observed in many more blood samples from patients with all types of leukemia.

The effect of sulfhydryl compounds was readily demonstrable on human plasma albumin obtained from Cutter Laboratories, Berkeley, Calif. The crystalline material was prepared by a cold ethanol fractionation procedure and was free of preservatives. Albumin dissolved in 0.1*M* phosphate buffer (*p*H 7.4) yielded a fine precipitate with ethanol. The precipitate was changed to a granular one by the addition of cysteine. The time required for this change to take place was dependent upon the concentrations of albumin and cysteine.

After serum albumin was treated at 5°C with performic acid (*1*) to oxidize sulfur to the sulfonic acid level, the change to a more granular precipitate upon the addition of cysteine to the aqueous ethanol suspension was not observed.

Another method for removal of sulfhydryl and sulfide groups was tested. Albumin in 0.1*M* phosphate buffer (*p*H 7.4) was treated at room temperature with excess cysteine (1 mole of albumin to 200 moles of cysteine), and the mixture was allowed to stand for 1½ hours. A twofold excess of N-ethylmaleimide was then added, and the mixture was allowed to stand at room temperature for 1½ hours. The addition of cysteine and N-ethylmaleimide was repeated in the same manner. After dialysis against frequent changes of phosphate buffer (*p*H 7.4) to remove excess reagents, three volumes of ethanol were added. A fine precipitate was obtained that did not show the rapid change expected upon the addition of cysteine.

The findings with ethanol denaturation of plasma proteins are similar to those obtained by others (*2, 3*) with heat and urea denaturation of plasma proteins. The mechanism proposed by Huggins *et al.* (*3*) to explain the catalytic effect of sulfhydryl compounds upon gel formation with heat and urea can be used to explain the ethanol precipitation phenomenon. It would appear that sulfhydryl compounds catalyze the polymerization of denatured plasma protein by increasing the extent to which intermolecular sulfide bonds are formed, and that the granular precipitates indicate extensive polymerization.

It now appears that the phenomenon of aggregation by the formation of intermolecular disulfide bonds may be associated with the action of many protein precipitants. Two distinctly different mechanisms can lead to this type of polymerization. The sulfhydryl-catalyzed reaction in which intramolecular bonds are broken with the formation of intermolecular bonds can take place anaerobically. Formation of intermolecular disulfide bonds by oxidation of sulfhydryl groups has also been described. The extent of polymerization by either mechanism appears to be determined in part by the number of sulfhydryl and disulfide bonds in the protein and the extent to which the protein precipitant changes the availability of these groups for interaction. Although denaturation may increase the extent of polymerization, it does not appear to be essential for aggregation. Straessle (*4*) has reported that a disulfide dimer of mercaptalbumin is formed without denaturation upon standing in cold aqueous ethanol (72 to 94 percent ethanol by volume). It was suggested that this dimerization may occur by the sulfhydryl-catalyzed type of reaction. Briggs and Wolf (*5*) observed polymerization, apparently without denaturation, by oxidation of soybean protein precipitates.

The polymerization process appears to be faster and more extensive with a protein precipitate. Straessle (*4*) has observed more rapid and complete oxidation of the solid mercury dimer of mercaptalbumin with iodine in aqueous ethanol. Briggs and Wolf (*5*) have observed extensive oxidation of soybean protein by air when precipitation was induced by cooling of the aqueous solution. Sayre and Hill (*6*) have reported polymerization of serum lactic acid dehydrogenase after precipitation with ammonium sulfate. An intermolecular disulfide bond stabilization of fibrin clots has been postulated to involve the sulfhydryl catalyzed type of reaction that follows an initial nonsulfide aggregation of fibrin monomer (*7*). A disulfide polymerization process was found by Deutsch and Morton (*8*) to take place with the globulins from macroglobulinemic serum isolated by precipitation techniques. The importance of the solid phase may lie in the fact that a more favorable orientation of sulfhydryl and disulfide groups is possible. The influence of a solid surface and sulfhydryl orientation on oxidation is illustrated by the work of Callaghan (*9*). Myokinase was found to have two sulfhydryl groups per molecule that underwent oxidation in glass or cellophane tubes that was not observed in plastic tubes or collodion tubes pretreated with hemoglobin.

Disulfide aggregation as discussed above should be clearly differentiated

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from other forms of aggregation that also involve sulfhydryl groups. Hughes (*10*) has described the formation of a mercury dimer of serum albumin, and Madsen, Cori, and Gurd (*11*) have shown an apparently new type of sulfhydryl aggregation of phosphorylase that is prevented by p-chloromercuribenzoate.

Huggins *et al.* (*12*) isolated serum albumin from normal individuals and from patients with malignant disease and found the sulfhydryl content to be the same in each group despite the fact that gel formation of plasma proteins in the latter group was abnormal. It does not appear probable that the plasma proteins from the leukemic patients have an abnormal sulfhydryl content since the ethanol precipitation phenomenon appears to depend upon the plasma nonprotein sulfhydryl level.

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"Slow" Potential Change in the Atrioventricular Node

Previous considerations of atrioventricular (A-V) conduction have been based on indirect physiological (*1*), clinical (*2*), and pharmacological (*3*) studies, but there has been no direct knowledge of intranodal events. An earlier report from this laboratory indicated the feasibility of recording from the buried portions of the A-V conduction tissue *in situ* (*4*). This technique has furnished some direct evidence about events in the A-V node (*5*).

As a small electrode is moved from the ventricle toward the atrium in the right and left bundles and then into the common bundle, the potential difference between this electrode and a distant point is symmetrical and biphasic, as would be expected in a "cable" of Purkinje fibers (*4*); this activity approaches closer in time to atrial depolarization as the electrode moves nearer to the atrium. The wave consists of a positive deflection (approaching activity), a rapid negative-going deflection (depolarization of cells near the electrode), and a terminal negative portion (receding activity) and appears earliest 30 to 40 msec after the firing of the atrial cells in the A-V nodal region. This discharge precedes the end of the P wave by about 15 msec in the dog. Similar deflections, indicating rapid depolarization of cardiac cells, were never recorded within this 30- to 40-msec interval. There thus existed a "silent" period during which no cellular firing was noted. One report of rapid potentials from the A-V node (*6*) does not place them in this silent period.

In some experiments, a slow potential change was recorded from electrodes in the A-V node during this "silent" period. When studied with direct-coupled amplifiers capable of working close to the theoretical noise limit, this change was found in the A-V node in 16 dogs.

Although many experiments were conducted on the heart *in situ*, examination of this potential required perfusion of the isolated heart, cutting of both conducting bundles, variable frequency stimulation of the sinoatrial node to produce first-degree A-V block, and exact histological localization of each electrode (Fig. 1, I). The apparatus has been described (*7*).

Potentials on an electrode in the mid A-V node are shown in Fig. 1, II. Following a stimulus to the sinoatrial nodal region (*S*), there is a large negative potential, the rapid deflection produced by depolarization of atrial cells in the A-V nodal region. Notches on the upstroke of this potential possibly result from firing of scattered cells in the atrionodal junction. The slow potential follows. It is initially positive, rising above the base line for about 20 msec, and then returns toward the base line. Fig. 1, II, shows changes that occur on increasing the rate of stimulation. The slow potential becomes prolonged, with a longer positive plateau and a long terminal negative phase.

One millimeter downstream (Fig. 1, III *c*), the slow potential has no negative phase; however, the negative-going phase coincides approximately with firing of the common bundle, 1 mm away (Fig. 1, III *b*). The slow potential varies in shape and magnitude, depending on electrode position.

In all studies, the slow potential has prolonged as the atrial stimulation rate has been raised from 2 to 3 per second to 10 per second. Concurrently, the interval between the atrial potential and the common bundle potential increases (Fig. 1, III, IV). At rates near 8 per second, the slow potential exhibits maximum prolongation, and complete (2 to 1) block occurs. During A-V rhythm (Fig. 1, IV), the slow potential precedes all other potentials and usually has a configuration differing from that during sinus rhythm.

The most likely picture of A-V nodal conduction which emerges from our study is the following: Atrial cells in A-V nodal region trigger atrionodal cells. These differ in anatomy from the atrial and the nodal cells (*8*). These cells activate the nodal cells, and the slow poten-

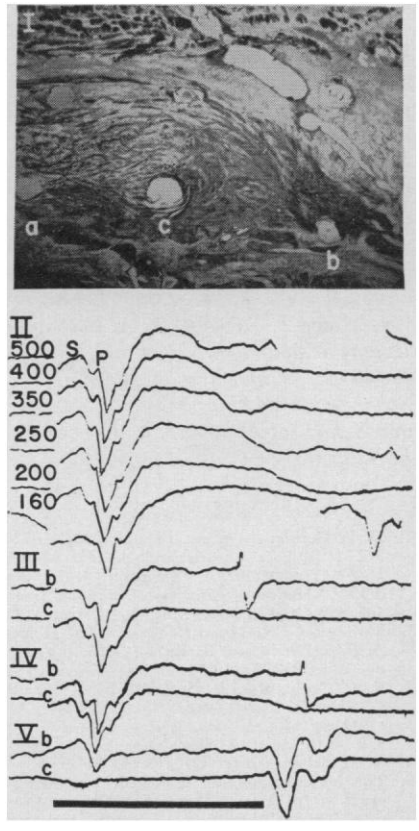

Fig. 1. (I) Histological section through the A-V node, showing locations of recording electrodes in *a*, upper A-V node; *b*, near origin of common bundle; and *c*, intranodal point midway between these. (II) Potentials recorded at point *a*; intervals between stimuli: 500, 400, 320, 250, 200, and 160 msec; *S*, stimulus artifact; *P*, auricular depolarization. At 160 msec, the common bundle did not follow the A-V node and the shape of the atrial potential was altered. The 500-msec record shows ventricular depolarization at the right. Right and left bundles had been cut, and the ventricles beat independently of the atria. (III) Potentials recorded at points *b* and *c* with interstimulus period of 400 msec. (IV) Potentials at *b* and *c* with interstimulus period of 200 msec. (V) Potentials recorded at *b* and *c* during A-V rhythm. (Parts of the auricular complex have been retouched to make the figure clearer.)