A Serum Protein System Affecting Contractility of the Frog Heart Present in Increased Amounts in Patients with Essential Hypertension

By Stephen Hady, M.D., and Edward Leonard, M.D.

Plasma samples from a group of patients with severe essential hypertension were formerly shown to have a cardiotonic action on the isolated frog heart. This effect is significantly greater than that observed with plasma from normal persons or those with other diseases. The material in plasma responsible for this action is a system of 3 proteins. These proteins have been distinguished by fractionation with sodium sulfate, and are found among the serum globulins. The protein, the concentration of which determines the activity of the whole system, is called component L. When applied to the frog heart it becomes strongly bound. Two other proteins are needed, one precipitated between 0 and 17 per cent sodium sulfate, the other between 22 and 30 per cent sodium sulfate. The 0 to 17 fraction is thought to require calcium for its activity, which is bound to the protein. Calcium can be released by the addition of component L in a reaction which is dependent on temperature, reversal being possible in high calcium concentrations in the cold. The protein system described causes contracture of frog heart muscle in the absence of ionic calcium, in contrast to other well-known contracture-causing agents which require the presence of free calcium for activity.

METHODS

Blood samples were drawn from human subjects or animals without regard to dietary intake. Either plasma or serum was used in these studies. In the case of plasma, high-potency heparin (100 mg./ml.) was added to blood to achieve a final concentration of 0.1 mg./ml. blood. Use of less concentrated heparin preparations which contained the same concentration of phenol preservative as the high-potency material resulted in the addition of enough phenol to damage the protein system. This problem was best avoided by performing isolation procedures on serum.

Bioassays were performed on hearts of Rana pipiens according to the procedure described earlier. The frogs were kept at 12 C. to minimize seasonal variation, and the sex was also noted since males are much more sensitive than females to the system under study. The heart was cannulated, removed from the frog, exteriorized over the area of the atria to stop spontaneous rhythmicity, and placed in a bath where electrical stimulation could be applied at a controlled frequency. The total volume of solution in the heart cannula was always 3.0 ml. During the assay of serum or protein fractions the usual bubbling of the test solution with a 97 per cent oxygen and 3 per cent carbon dioxide gas mixture was stopped in order to avoid excessive foaming. The action on the isolated heart of the protein system under study is roughly comparable to that of cardiac glycosides: in low concentrations the stimulus frequency needed to achieve a given twitch tension is decreased, and at high concentrations contracture occurs. When different sera were compared for protein system activity, subcontracture concentrations were used; the greater the activity in a given sample, the more marked was the effect on the tension-stimulus frequency relationship. This is shown in figure 1, where continuous tracings of isometric twitch tension developed by
Fig. 1 Left. Action of serum on isolated frog heart. Original continuous tracing in which height of lines indicates twitch tension and numbers refer to time intervals in sec. between stimuli. Each tracing is from a different frog heart. A. First section, control in Boyle-Conway solution; second section, 1.0 ml. normal human plasma added. B. First section, control; second section, 0.66 ml. hypertensive human plasma. C. Control, followed by 1.0 ml. hypertensive human plasma.

Fig. 2 Right. Effect of normal serum on fresh heart compared to heart sensitized with 1.5 ml. dog component L. A. Fresh heart, 1.0 ml. normal serum added at arrow. B. Sensitized heart, 0.5 ml. normal serum added at arrow.

Essential changes in the tension-stimulus frequency relationship occur. Figure 1B is a comparable record of another heart showing a control strip followed by the effect of 0.66 ml. plasma from a patient with severe essential hypertension. The serum causes twitch tension to be maintained at near maximal values even when the stimulus frequency interval is as long as 60 seconds. Figure 1C shows the effect on a third heart of 1.0 ml. plasma from the same patient. This amount of plasma causes contracture, characterized by a drop in twitch tension and a rise in base line to the point where there occurs no contractile response to electric stimulation.

During isolation procedures the concentration of activity in any fraction was estimated by determining the minimum amount of sample in the 3.0 ml. volume needed to cause contracture. For experiments with calcium-free solutions, the heart was pumped mechanically by a device that caused a cyclic change in the air pressure acting on the external surface of the ventricle. Mechanical pumping was used since no muscular contractions occur in the absence of calcium, and the alternate increase and decrease in the volume of the ventricular cavity promotes exchange of substances between the perfusion fluid and the heart muscle.

Protein fractions from serum were made by sodium sulfate precipitation. Analytical Na₂SO₄ was dissolved in deionized distilled water containing .005M CaCl₂. The material to be precipitated was placed in a cellulose casing (Visking Corp. size 3/8-inch flat) and dialyzed against the sodium sulfate solution in a lucite cylinder placed on a rocking dialyzer so that both inside and outside solutions were mixed. This operation was carried out at 25 C. when sodium sulfate solutions up to 25 per cent (wt./vol.) were used. Temperature was maintained at 30 to 32 C. in the preparation of fractions with 30 per cent Na₂SO₄ or more because of the limited solubility of Na₂SO₄ in water. For this purpose a small rocking dialyzer was set up in a Fisher Senior Isotemp oven. The protein fractions were separated after transfer to 15 ml. lueroid tubes, by spinning at maximum speed for 10 to 15 min. in a temperature-controlled centrifuge (Internat. Refrig. Centr., Model PH-1, head no. 269). For operation of this centrifuge at 30 C., the chamber was preheated by steam. The required temperature was kept constant during spinning of the sample by the balance of heat produced by friction and the cooling by refrigeration. Precipitates made by dialysis against sodium sulfate solutions up to 25 per cent spin down readily to form a well-packed pellet. The precipitate made with 30 per cent Na₂SO₄ rises during spinning and forms a
solid disk on top of a clear solution. The precipitate can be separated from the underlying fluid by first pricking a hole in the bottom of the lusteroid tube and then cutting off the tube below the level of the precipitate. Precipitates made at sodium sulfate concentrations between 25 and 30 per cent cannot be centrifuged satisfactorily, because of insufficient difference in density between the solid and liquid phases.

RESULTS

Preliminary Evidence That System Is Protein. Preliminary experiments were performed on plasma from patients with hypertension, such plasma containing more activity than that from normal subjects. Exhaustive extractions with chloroform-methanol mixtures, followed by chromatography on a Florisil column yielded only a small percentage of the activity present in the original plasma. Slow addition to plasma of acetone or ethanol at 0 C. for achieving a final concentration of 20 per cent caused a disappearance of the original plasma activity. These results suggested the possibility that the activity occurs in the protein fraction of the plasma, an idea that was further supported by the findings of lability on heating at 65 C. for 10 min. in an atmosphere of nitrogen. More evidence was provided by the following experiment. Plasma was filtered under pressure through a collodion membrane of a pore size which retained all but the smallest proteins (protein concentration in the filtrate, determined by ultraviolet light absorption at 280 mμ was 82 mg. per cent). Approximately half the original volume of the plasma was filtered, and then both the filtrate and the concentrated plasma remaining inside the collodion bag were tested. Full activity was found in the concentrated plasma, whereas none was found in the ultrafiltrate. These findings lent support to the idea that the activity was in the protein fraction of plasma.

Evidence that System Comprises More than One Factor. In the course of performing bioassays on the system under study it was found that if a plasma sample was added to the isolated heart and a certain activity were measured, all activity disappeared in 10 to 15 min. on a washing with Boyle-Conway solution. However, addition of the same amount of fresh plasma to the heart a second time had a greater effect than did the first dose, and there was a progressive increase in activity to the point of contracture with successive doses of plasma, despite the fact that all apparent activity could be washed out between doses. In other words, application of plasma altered the response of the heart to the next dose, although the effect could not be detected in the washed heart between applications. It seemed reasonable to suppose, therefore, that the activity of the system is due to two components, one strongly bound to the heart, the other easily removed by washing. Thus the progressive alteration in the response of the heart to successive applications of plasma would be due to the accumulation of the binding substance. The disappearance of the plasma response between applications is attributed to the removal of the nonbinding part of the system, which will subsequently be called the "washable fraction." A heart which has accumulated the binding substance, according to this hypothesis, is called "sensitized." The binding is so strong that no significant loss occurs from a sensitized heart in Boyle-Conway solution over a period of 24 hours. The tension-stimulus frequency relationships of a washed sensitized heart in Boyle-Conway solution resemble those of a heart that has never been exposed to the plasma system since the washable fraction is absent. The addition to a sensitized heart of the washable fraction in the form of a small amount of plasma from a normal subject causes the appearance of activity, a much greater activity than would be expected from the same amount of normal plasma added to a fresh unsensitized heart. These phenomena are demonstrated in figure 2. The first section of tracing A shows the tension-stimulus frequency relationship in a normal heart. Maximal tension occurs with stimuli spaced 5 sec. apart, tension drops when the stimulus frequency interval is increased to 10 sec. and rises again when the 5 sec. frequency interval is resumed. One ml. normal serum, applied at the beginning of
the second section of the tracing, has little
detectable effect. Tracing B, on the other
hand, is taken from a washed sensitized heart.
It appears comparable to the fresh heart, but
when the washable fraction was supplied, by
the addition at the arrow of 0.5 ml. normal
serum, contracture occurred, a phenomenon
not caused by twice this amount of normal
serum added to the fresh heart of tracing A.

Isolation of the Binding Component. The
binding fraction which sensitizes the heart is
called component L, because it is the limiting
factor, the concentration of which determines
the activity of the whole system in human and
animal bloods. Any L fraction obtained by
isolation procedures free of the washable fraction
should have no detectable effect on a fresh heart, but should alter it so that subse-
quent addition of the washable fraction causes
activity. To assay a component L fraction,
one determines the minimum amount affecting
the heart to the extent that contracture occurs
with the subsequent addition of 0.5 ml. nor-
mal serum (which supplies the washable
fraction) without adding significantly more L to
the medium (fig. 2).

The starting material for the preparation of
component L is plasma with high activity. Blood
from either hypertensive human beings or normal
dogs is suitable for this purpose. The present
method of preparing an L fraction is as follows:
To 20 ml. serum at 25 C. are added 20 ml. 25
per cent Na2SO4. The mixture is transferred to
3/8-inch (flat) cellulose casings and dialyzed 1
hour against 400 ml. of 25 per cent Na2SO4 so
that the concentration of Na2SO4 at the end of
equilibration is approximately 24 per cent. The
precipitate is separated by centrifugation for 30
min. at 25 C., dissolved in approximately 5 ml.
of demineralized distilled water, and dialyzed in
3/8-inch cellulose casings against 600 ml. of water
at 4 C. for 1 hour to free the solution of Na2SO4.
The fraction can be used in this form (after
equilibration against Boyle-Conway solution), but
cannot be depended upon to last more than 24
hours in the cold or in the frozen state. Therefore,
after the 1 hour dialysis against water, the dialysis
bag is transferred to 100 ml. of 55 per cent
glycerol in water at 4 C. and equilibrated 1 to 2
hours. The contents of the bag are then extruded
into a graduated centrifuge tube and stored at
-20 C. The L fraction stored at -20 C. in glycerol
has been found not to lose activity over a period
of many weeks. An amount of this material cor-
responding to 1.0 to 2.0 ml. of dog or hypertensive
human serum is sufficient to sensitize a heart so
that subsequent addition of the washable fraction
causes prompt and complete contracture. Sensi-
tizing aliquots of component L are prepared by
measuring a given amount of the stored material
into a 3/8-inch cellulose casing and dialyzing
against 100 volumes of Boyle-Conway solution
for 45 min. The material is then made up to a
final volume of 3.0 ml. with Boyle-Conway solution
and added to the heart. The heart is stimu-
lated once every 5 sec. and after approximately
15 min. the solution is washed out. The heart
is thus sensitized.

Some purification of L can be accomplished by
dialyzing the material against ion-free water at
4 C. In 2 to 3 hours a euglobulin precipitate
appears, L activity remaining in the supernatant.

The pH stability of the component L prepara-
tion was studied from samples dialyzed against
buffer solutions at 4 C. for a 2 hour period, and
then restoring to physiological pH by dialysis
against Boyle-Conway solution. Most of the ac-
tivity is destroyed at pH 4.0, whereas most of
it is retained at pH 5.0 (acetate buffers). On the
alkaline side, activity is retained in carbonate-
bicarbonate buffers as high as pH 10.0. The range
of stability is narrowed if the pH is changed by
direct slow addition of acid or alkali without the
use of a dialysis membrane.

Studies on the Washable Fraction. It was
observed early in the study that dialysis of
serum from hypertensive patients against
large volumes of water at 18 to 25 C. for 2
hours was associated with a loss of washable
fraction activity, i.e., inability of as much as
1.0 ml. of the dialyzed serum to cause contrac-
ture when added to a sensitized heart (fig.
2B). This occurred occasionally, but much
less frequently with normal serum. If the
loss was due to movement of the washable
fraction out of the cellophane dialysis bag, it
would have been expected that the material
was also free to pass through a large-pore el-
lodion membrane during ultrafiltration of
serum. However a serum ultrafiltrate pre-
pared as described in the first section under
"Results" exhibited no washable activity.
Therefore it seemed likely that the washable
fraction was not free to pass through a col-
lodion or cellophane membrane, and that
the loss of activity on dialysis was due either to
the dissociation of a dialyzable factor from a nondialyzable moiety or to the destruction of a nondialyzable molecule. The loss of activity could be diminished if dialysis were carried out against a solution previously dialyzed against 3 volumes of calf serum so that it contained about 75 per cent of the original concentration of the dialyzable components of serum. Further investigation showed that the loss on dialysis against water could be prevented simply by the inclusion of 0.005 M CaCl₂ in the dialyzing solution. Washable activity could not be supplied by calcium salts alone. Attempts then were made to isolate the washable fraction, based on the probability that it was a nondialyzable protein either containing calcium or maintained in a stable state by ionic calcium.

It was found that all washable activity could be recovered in a protein fraction that included the serum globulins and a small amount of albumin. A pure albumin fraction exhibited no activity. The isolation procedure was as follows: To 12 ml. serum from a normal human subject were added 12 ml. of 0.005 M CaCl₂. This mixture was dialyzed at 30 to 32 C. for 1 hour against 600 ml. of 31.2 per cent Na₂SO₄ with or without 0.005 M CaCl₂. The sodium sulfate concentration at the end of the dialysis period approached 30 per cent. The contents of the dialysis casing were then placed in a lusteroid test tube preheated to 30 C. and spun 15 min. The cake of precipitate at the top of the clear underlying fluid was separated according to the procedure outlined in the “Methods” section, dissolved in approximately 3 ml. of 0.005 M CaCl₂ and dialyzed against 100 volumes of 0.005 M CaCl₂ at 4 C. for 1 hour. For testing, the material was then dialyzed against Boyle-Conway solution at 4 C. for 45 min. An aliquot was brought up to a volume of 3.0 ml. and added to a washed sensitized heart. By varying the amount of aliquot, the minimum
necessary for contraction was determined. An amount equivalent to 0.1 to 0.5 ml. of serum (depending on the sensitivity of the frog heart) in the 3.0 ml. volume of test solution caused contraction. Essentially no loss of activity occurred during precipitation with 30 per cent Na₂SO₄. On the other hand, the material obtained by 25 per cent sodium sulfate precipitation under similar conditions contained much less than the total activity. Since this suggested that a large fraction of the activity was precipitated between 25 and 30 per cent sodium sulfate concentrations, it seemed likely that further purification could be achieved by discarding the proteins that were precipitated at lower concentrations of Na₂SO₄. The following experiment, however, forced a revision of this viewpoint. A 30 per cent sodium sulfate fraction from normal serum was made according to the method described above and is illustrated by figure 3. This protein material was redissolved and equilibrated against 17 per cent Na₂SO₄. The precipitate (the 0 to 17 cut) was separated by centrifugation. The sodium sulfate concentration in the supernatant was brought to 22 per cent by dialysis, and the new precipitate (the 17 to 22 cut) was separated. Finally, this supernatant was brought to a concentration of 30 per cent sodium sulfate yielding a precipitate (the 22 to 30 cut). Each of these 3 fractions was equilibrated against Boyle-Conway solution and activity, as indicated by the minimal amount needed to cause contraction in a sensitized heart, was determined. Two-tenths milliliter of the original 0 to 30 cut caused contraction. In contrast, no contraction occurred with the addition of 0.8 ml. of the 0 to 17 cut, 1.3 ml. of the 17 to 22 cut, or 2.5 ml. of the 22 to 30 cut. The inactivity of the 22 to 30 cut was particularly surprising in view of previous experiments that had suggested that a large proportion of the activity was precipitated between 25 and 30 per cent Na₂SO₄. Accordingly, a mixture of 0.4 ml. of the 0 to 17 cut and 0.4 ml. of the 22 to 30 cut was added to a sensitized heart, and contraction occurred. This led to the unavoidable conclusion that 2 protein components are required for activity of the washable fraction.

Outlined below is the current procedure for isolating the 2 components of the washable fraction. From 18 ml. of fresh normal human serum a 0 to 30 per cent sodium sulfate fraction is prepared as described above. After centrifugation, the precipitate is dissolved in 0.065 M CaCl₂ at 23 °C, the volume being adjusted to 18 ml. This solution is dialyzed against 600 ml. of 22.6 per cent Na₂SO₄ for 1 hour at 23 °C. After a 10 min. centrifugation, both the precipitate and the supernatant are further simultaneously processed as follows: The precipitate is dissolved in water, and after volume adjustment of the solution to 18 ml. it is dialyzed against 17.4 per cent Na₂SO₄ for 1 hour, the resulting precipitate being the 0 to 17 fraction. At the same time the supernatant is dialyzed against 600 ml. of 31 per cent Na₂SO₄ for 1 hour at 30 to 32 °C to precipitate the 22 to 30 fraction. After centrifugation each fraction is dissolved in about 3 ml. of 0.065 M CaCl₂ and 0.1 M NaCl and is dialyzed against 600 ml. of a solution of the same composition at 4 °C for 1 hour, to remove the sulfate ion. Then, after equilibration against Boyle-Conway solution, the components are ready for testing.

The separated fractions are very unstable and usually were used for experiments the same day. Mixing of the 2 separated fractions stabilizes both.

The Effect of Calcium Ions on the Washable Activity. If samples of serum are dialyzed against large volumes of water, some loss of washable activity occurs, but the extent of this loss varies widely in different sera. Samples from patients with hypertension, for example, usually completely lose activity on dialysis against 100 volumes of water at 25 °C for 2 hours, whereas sera from normal subjects infrequently exhibit loss under these conditions. Since one difference between these 2 groups is a higher concentration of component L in the sera of hypertensives, the effect of increasing the amount of component L in normal serum was studied. It was found that a normal serum exhibiting no detectable loss of activity under the above dialysis conditions did lose activity on dialysis when component L was added. This phenomenon is highly dependent on temperature, in that the extent of loss in a given period of time can be diminished greatly by lowering
Fig. 4. Action of various contracture-causing agents on heart in calcium-free medium. Original tracings from hearts in calcium-free Boyle-Conway solution. Absence of tension is indicated by the horizontal line, interrupted periodically by artifact (vertical lines) caused by mechanical pumping. Agents tested were added at arrows. Each tracing is from a different heart. A. Normal heart, 1 ml. calcium-free normal serum added at arrow. B. Sensitized heart, 1 ml. calcium-free normal serum added. C. Normal heart, 50 μg./ml. digitonin. D. Normal heart, 2 μg./ml. strophanthinidin.

The loss of activity on dialysis, even in sera containing high concentrations of component L, can be prevented if dialysis is carried out against 0.005 M CaCl_2 instead of against water. Furthermore, in the case of serum samples inactivated by dialysis, reactivation can be accomplished in a variety of ways having in common the addition of calcium ions at low temperature. In early experiments, before the nature of the reactivating material was known, calcium was supplied in the form of fresh serum, dialyzed against an equal volume of the inactive material at 4 C. Later, the inactive samples were restored by dialysis against 0.005 M CaCl_2 or by direct addition of CaCl_2 to achieve a concentration of 5mM/L, both procedures being carried out at 4 C. When calcium was added directly, the samples were allowed to stand 15 min. in the cold before testing. In summary it was demonstrated that under the appropriate conditions activity could be lost by dialysis against calcium-free solutions, and could be regained by addition of calcium at low temperature.

These experiments led to the following hypothesis. One or both of the protein fractions comprising the so-called washable fraction (either the 0 to 17 or the 22 to 30 cut) contain calcium bound strongly, and the calcium-protein complex is necessary for activity of the system. Calcium is freed from the complex by means of the fraction containing component L in a reaction which occurs readily at 25 C. in the absence of ionic calcium but is greatly slowed at 4 C. The calcium-free protein may recombine with calcium again in the presence of the free ion at low temperature.

The following experiment was designed to determine which component of the washable fraction (0 to 17 or 22 to 30) exhibited the calcium sensitivity. One half milliliter of normal serum was mixed with 0.04 ml. component L and dialyzed against 100 volumes of water at 31 C. for 1 hour, whereas 0.5 ml. of the same serum +0.6 ml. component L remained active under similar dialysis conditions at 4 C.

The Effect of Glycerol on the 22 to 30 fraction. When 0.25 ml. glycerol was added, at room temperature, to 1.0 ml. of serum and this mixture dialyzed against 10 volumes or more of 50 per cent glycerol for at least 3 hours at 4 C., disappearance or marked diminution in 22 to 30 activity occurred. The loss in glycerol could be prevented if at least 0.1M NaCl, Na_2SO_4, MgCl_2 or MgSO_4 were present.
Furthermore, when a sample inactivated by the above procedure was dialyzed against 50 per cent glycerol containing any one of the above salts (0.2 M), activity was regained. 

**Physiological Experiments.** Since the results of previous experiments suggested that calcium was released from the 0 to 17 protein in the presence of the component L fraction, it was of interest to see what the effect of this system might be on a frog heart deprived of calcium. Therefore the usual Boyle-Conway solution perfusing the frog heart was replaced by a solution of the same composition except for the absence of calcium ions, after which the twitch tension declined to zero or near-zero levels within a few seconds. Electrical stimulation was continued at 5 sec. intervals, but because of the absence of contractile response the heart was pumped mechanically for an adequate equilibration between the tissue and the perfusion solution. After it was certain that not the slightest response to electrical stimulation could be detected, 1.0 ml. of serum mixed with versene and then dialyzed against large volumes of 0.1 M NaCl to eliminate the calcium-versene complex, and 2.0 ml. of calcium-free Boyle-Conway solution were added to the heart. This mixture, which was completely free of ionic calcium, had no effect. However, when the same solution was added after the heart had been sensitized with component L, contracture occurred without reappearance of twitch tension (figure 4A and 4B).

It was decided to recheck this point with representatives of 2 of the best known groups of contracture-causing materials, the saponins and the cardiac aglycones. From the saponins digitonin was chosen. A solution of 100 mg./L. was made up in 0.08 mM versene to remove traces of calcium contained in the digitonin preparation. The frog heart was perfused with a calcium-free solution until no twitch tension could be detected. At this point a mixture of 1.5 ml. of the digitonin solution and 1.5 ml. of a twice concentrated calcium-free perfusion fluid was added to the heart. No contracture occurred (fig. 4C). Addition of ionic calcium to the medium caused immediate contracture. Likewise, the addition of a contracture-causing concentration of strophanthidin (2 μg/ml.) had no effect in the absence of calcium (fig. 4D).

**Comparative Studies of the System in different Species.** Sera from dog, rabbit, monkey, guinea pig, hen, rat, cat, and sheep were tested for the activity under study. High activity was found in the first 3 animals listed; it was present but low in the others. Normal man falls into the low range; certain hypertensive patients are as high as any found among the other species. Component L and the washable fraction from different species can be combined to give activity. The addition of as much as 2 ml. of frog serum to a sensitized heart has no effect. For practical purposes, the frog does not appear to possess the protein system under study.

**Discussion**

The characterization of the system of plasma proteins presented in this paper was facilitated by the quantitatively measurable action of the system on the isolated heart of the frog. The action on the frog heart is cardiotoxic, similar in some respects to that of the cardiac glycosides. Both the glycosides and the protein system can restore the tension of a hypodynamic frog heart to that of the freshly excised state, can further increase contractility beyond this point as indicated by maintenance of maximum twitch tension at slow rates of stimulation, and will cause contracture if present in toxic concentrations (fig. 1).

The action on the frog heart thus provided the basis for a reliable bioassay, but afforded no certain insight into the physiological significance of the protein system. Had it been tested on some other organ of the frog an effect might likewise have been found, just as, in the case of epinephrine, activity on more than one tissue can be demonstrated. However, the effects on the frog heart, combined with the finding of high activity in hypertensive patients, make it reasonable to search for a physiological site of action in either the heart or the blood vessels.
Evidence has been presented in this paper that the plasma system under study is composed of 3 distinct proteins. Each of these has been obtained in crude fractions, and no purification has been attempted beyond that necessary to establish the existence of the 3 separate components. For example, despite the fact that the component L preparation comprises a large part of the serum globulins, it is free of washable fraction activity because precipitation in 24 per cent Na₂SO₄ leaves most of the 22 to 30 component in the supernatant, and the small amount that is precipitated is inactivated during the subsequent dialysis against salt-free glycerol. Furthermore, although the 0 to 17 component is precipitated by 24 per cent Na₂SO₄, the high concentration of component L causes loss of calcium and therefore inactivation of the 0 to 17 fraction.

Each one of the proteins under study is very sensitive to chemical manipulation, and even precipitation by the gentle procedure of salting out may result in great losses if neutral (NH₄)₂SO₄ rather than Na₂SO₄ is used. Among the 2 washable fraction proteins, little is known about the 22 to 30 component beyond the fact that it is needed for the activity of the whole system. Inactivation of the 22 to 30 component occurs on dialysis in the presence of glycerol and can be prevented or reversed by a variety of inorganic ions at a concentration of 0.2 M in glycerol. The non-specificity of the salt effect suggests that the inactivation may represent a reversible denaturation.

The reasons for believing that calcium is bound to the 0 to 17 component are that the washable activity of serum in the presence of component L disappears on dialysis against calcium-free solutions, is retained if 0.005 M CaCl₂ is present in the dialyzing solutions and can be restored after dialysis against calcium-free solutions by the addition of calcium or by the addition of fresh component 0 to 17. Several lines of evidence indicate that the binding between calcium and the 0 to 17 protein is different from that between, for example, calcium and serum albumin. Loss of activity (and therefore loss of calcium) is highly temperature dependent, in contrast to the dissociation of calcium from albumin which exhibits no temperature dependence from 0 to 25 C. Furthermore, loss of activity is enhanced by the addition of component L, which suggests that the calcium protein complex is acted on by this component. The strength of the complex is further demonstrated by the fact that dialysis of serum against several changes of large volumes of calcium-free solutions at 4 C. for 5 to 15 hours does not affect the activity of the protein system, and 200 to 400 µM/L of calcium remain bound in such dialyzed samples, as determined by a bioassay method a report on which is to be published.

From the above discussion it is clear that one member of the protein system contains strongly bound calcium, and that another member, which binds tightly to the isolated heart, is capable of releasing this calcium. The biological effect of the system can be reproduced in part (alteration of stimulus frequency tension relationship) by an increase of the concentration of ionic calcium in the perfusion medium. Moreover, the need of calcium for shortening of the contractile protein in intact muscle has been underlined by the demonstration that a heart in a calcium-free medium is incapable of developing tension, even in the presence of large concentrations of strophanthidin or digitonin. In the face of the general rule, then, that heart muscle does not shorten without calcium ion; even in the presence of powerful contracture-causing agents, it has been found that the protein system will cause contracture in the absence of ionic calcium. All these facts force consideration of the hypothesis that the biological activity of the protein system on the frog heart may be achieved by delivering calcium from component 0 to 17 to the contractile mechanism.

**SUMMARY**

The isolation and characterization of a plasma protein system, found to be increased...
above normal in a group of patients with severe hypertension, has been described.

The methods for isolation are based on the action of this system on the isolated frog heart; alteration of the stimulus frequency-tension relationship occurring first, followed by contracture at higher concentrations.

The system was found to consist of 3 globulins, all of which are needed for activity. One protein, component L, binds strongly to the frog heart. The other two, which do not bind, were named according to the concentration range of sodium sulfate needed to precipitate them, i.e., the 0 to 17 and the 22 to 30 fractions.

Arguments were presented to support the hypothesis that the activity of the 0 to 17 fraction depends on the presence of bound calcium which is not removed from the protein by prolonged dialysis but which can be released in the presence of component L by a reaction which is temperature sensitive. Reactivation of the 0 to 17 protein can be accomplished in the presence of high calcium concentrations in the cold.

The apparent importance of calcium for the 0 to 17 protein led to physiological experiments to determine the effect of the whole system on the isolated frog heart in the absence of free calcium. Under these conditions the system could still cause contracture, in contrast to two well-known contracture-causing agents, strophanthidin and digitonin, which require the presence of ionic calcium for activity.

It was pointed out that the action of the protein system on the frog heart provided the basis for a bioassay which led to the characterization of the 3 separate components, but that no conclusions about the physiological role in mammals can be drawn therefrom. A site of action on either heart or blood vessels is reasonable, however, in view of the effects on the frog and the increased activity of the system noted in hypertensive humans.

**Summary in Interlingua**

Es describite le isolation e le characterisation de un systema de proteinas del plasma, le qual se monstrava presente in quantitates plus que normal in gruppo de patientes con sever grados de hypertension.

Le methodos de isolation es basate super le action que iste systema exerce super le corde del rana: Occurre primo un alteration del relation inter frequentia stimulatori e tension e postea contractura (con le attingimento de plus alte concentrationes.

Esseva trovate que le systema consiste de 3 globulinas, le quales es omnes necesse pro le activitate. Un del 3, componente L, se liga fortemente al corde de rana. Le altere 2 non ha iste characteristica. Illos esseva identificate secondu le scala de concentrationes de sulfato de natrium que es requirite pro lor identification. I.e. le fraction 0 a 17 e le fraction 22 a 30.

Es presentate argumentos in supporto del hypothese que le activitate del fraction 0 a 17 depende del presentia de calcium in forma ligate a illo. Isto non se detacha ab le proteina per periodos prolongate de dialyse, sed in le presentia del componente L illo es liberate in un reaction que es thermo-sensibile. Reactivation del proteina 0 a 17 es effectuabile in le presentia de alte concentrationes de calcium a temperaturas frigide.

Le apparente importancia de calcium pro le activitate del proteina 0 a 17 suggereva expeimentos physiologic con le objectivo de determinar le effecto del integre systema super le isolate corde de rana in le absentia de calcium libere. Sub iste conditiones le systema reteneva su capacitate de causar contracturas, per contrasto con le ben-cognoscite agentes contracturogene, strophantidina e digitonina, que require le presentia de calcium ionic pro lor activitate.

Es signalate que le action del systema de proteinas super le corde de rana provideva le base pro un bioessay o que permiteva le characterisation del 3 separate componentes, sed il es necessse notar que isto non poteva ducer a conclusiones con respecto al rolo physiologic que le systema joca in mammalianos. Nonobstante, il pare plausibile supponer un sito de action in le corde o le vasos de sanguine, viste
le effectos in le corde de rana e viste le augmentate activitate de illo in patuentes hypertensive.

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A Serum Protein System Affecting Contractility of the Frog Heart Present in Increased Amounts in Patients with Essential Hypertension

STEPHEN HAJDU and EDWARD LEONARD

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