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The Activation of Plasminogen by Staphylokinase: Comparison with Streptokinase

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Plasminogen, the precursor of the proteolytic and fibrinolytic enzyme of blood, is activated by various substances, including the bacterial activators streptokinase (Milstone, 1941) and staphylokinase (Lack, 1948; Gerheim, Ferguson, Travis, Johnston & Boyles, 1948). Some animal plasminogens which are unaffected by streptokinase alone are activated by a mixture of streptokinase and human globulin (Müllertz & Lassen, 1953; Sherry, 1954). Müllertz has postulated the existence in human serum of a proactivator which is converted by streptokinase into an activator of plasminogen (Müllertz & Lassen, 1953; Müllertz, 1955). Staphylokinase activates a wider range of animal plasminogens (Gerheim & Ferguson, 1949) and may therefore not require proactivator. The activation of human and dog plasminogens by staphylokinase has been studied by Lewis & Ferguson (1951), Hayashi & Maekawa (1954) and Celander & Guest (1959).

This paper describes the activation by staphylokinase of human, rabbit and guinea-pig plasminogens. When sufficiently concentrated, staphylokinase acted as rapidly as streptokinase. It did not, however, activate ox plasminogen and it was unaffected by the addition of proactivator.

EXPERIMENTAL

Buffer. Palitzsch's borate buffer, pH 7·4, prepared as described by Norman (1957), was used throughout.

Casein. Light white soluble casein (British Drug Houses Ltd.) was used without further purification.

Human plasminogen. This was purified by the procedure of Kline (1953). This preparation was also used as a source of proactivator.

Euglobulin. Human and animal euglobulins were precipitated from serum as described by Norman (1957). They were resuspended in borate buffer in the original serum volume or, with ox euglobulin, in 0·4 vol.

Streptokinase. Dornokinase (Burroughs Wellcome and Co.) was used.

Activation and assay of plasminogen. This was carried out by the method described by Norman (1957). Perchloric acid was used instead of trichloroacetic acid to precipitate casein (Müllertz, 1955). This modification raised the extinction at 280 mμ due to the products of casein digestion by 50%. Measurements of E were made in the Unicam spectrophotometer model SP. 500. Activity was expressed in terms of a unit which gives, under the conditions of the test, an increase in E at 280 mμ of 1·00/min. of digestion.

Assay of staphylokinase. Several concentrations of the staphylokinase to be assayed were incubated for 30 min. at 37° with 50×10^{-3} unit of human plasminogen, in 1·1 ml. of borate buffer, pH 7·4. A portion (1 ml.) of 4% (w/v) casein was added and incubation continued for 30 min. Casein was then precipitated with 3 ml. of 10% perchloric acid and E at 280 mμ of the digestion products was measured. The staphylokinase activity was determined by interpolation. A unit of staphylokinase was defined as that activity which gives rise, under these conditions, to an increase in E of 0·300. This unit was approximately equal to one-third of a Christensen unit of streptokinase (Christensen, 1949) (Fig. 1). In the experimental results to be described, streptokinase activity also is expressed in these extinction units.

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Preparation of staphylokinase. Strains of *Staphylococcus aureus* were selected for their fibrinolytic activity (Lack, 1957). A volume (450 ml.) of casein hydrolysate medium (Duthie & Haughton, 1958) in a penicillin flask was inoculated with 50 ml. of an overnight-shaken culture in digest broth and incubated at 37° on a horizontal shaker rotated at 45 rev./min. for 7 hr. Most of the organisms were removed by centrifuging. The remainder were killed by heating at 75° for 30 min.

Batches with a high staphylokinase activity, as measured by the caseinolytic method, were concentrated as follows. About 50% of the inert protein was precipitated by adjusting the pH to 3·3 with 10 N-HCl, a pH meter with glass electrode (Cambridge pH Indicator) being used. After centrifuging, the supernatant was dialysed against distilled water for 48 hr. at 5°, with Visking tubing as the membrane. For 4 l. of the supernatant, about 60 l. of water was used and this was changed four times. When necessary N-HCl was added to bring the final pH to 4·2. The staphylokinase precipitated by this procedure was centrifuged and dissolved in 10 ml. of borate buffer/l. of starting material. The yields varied greatly but the average was about 35%. This material had an activity of 2–4 units/μg. of protein. (Protein was determined by the method of Lowry, Rosebrough, Farr & Randall, 1951.)

RESULTS

Activation of human plasminogen by staphylokinase

Kinetics of activation. Purified human plasminogen was activated by staphylokinase, the rate of activation depending on the concentration of staphylokinase (Fig. 2). Because of the instability of the plasmin formed, the maximum activity

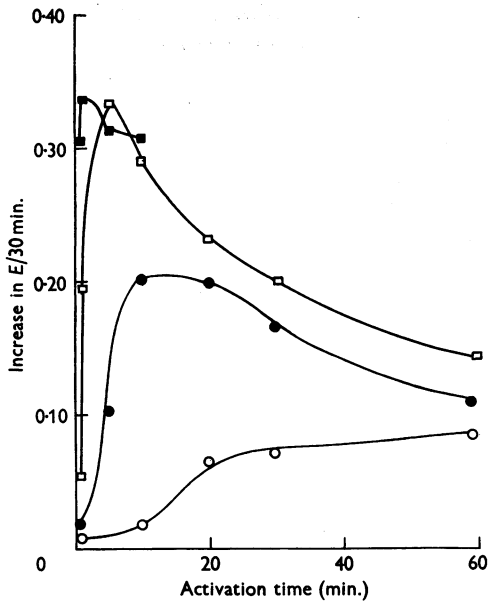

Fig. 2. Activation of purified human plasminogen by staphylokinase. Plasminogen ($11·2 \times 10^{-3}$ unit) was incubated for the times shown with 0·5 (○), 5 (●), 50 (□) and 500 (■) units of staphylokinase in a total volume of 1·1 ml. A portion (1 ml.) of 4% (w/v) casein was then added and the proteolytic activity determined as described in the Experimental section and expressed as the increase in E at 280 mμ during 30 min. of casein digestion.

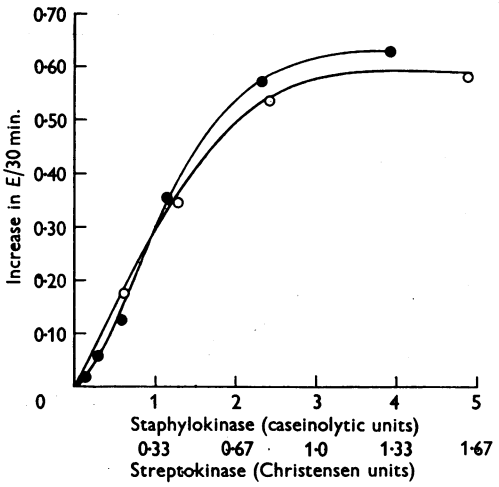

Fig. 1. Assay of staphylokinase and the relationship between casein digestion and Christensen units of streptokinase activity. Streptokinase (○) and staphylokinase (●) were assayed as described in the text. The Christensen units of activity of the streptokinase used were supplied by the manufacturer.

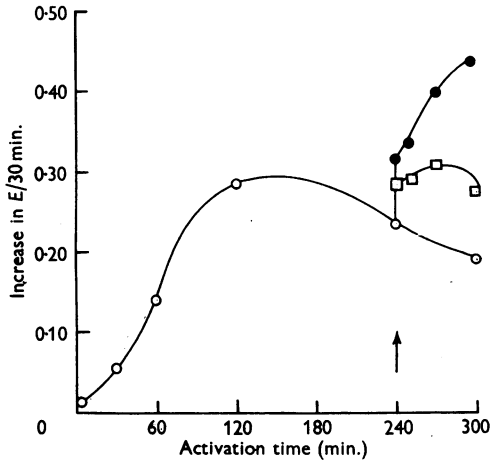

Fig. 3. Stability of staphylokinase during activation of human plasminogen. Plasminogen (76×10^{-3} unit) was incubated with staphylokinase (0·3 unit). After 4 hr., 30×10^{-3} unit of plasminogen (●) or 0·3 unit of staphylokinase (□) was added. Other conditions were as described for Fig. 2.

Table 1. *Inhibition of staphylokinase and streptokinase by casein*

Human plasminogen ($11{\cdot}2 \times 10^{-3}$ unit) was activated by 5 units of staphylokinase or streptokinase in the presence or absence of the casein substrate. Conditions were as described for Fig. 2.

Incubation time (min.)		Activity (increase in E)	
Before adding casein	After adding casein	Staphylokinase	Streptokinase
10	30	0·202	0·165
0	40	0·010	0·070

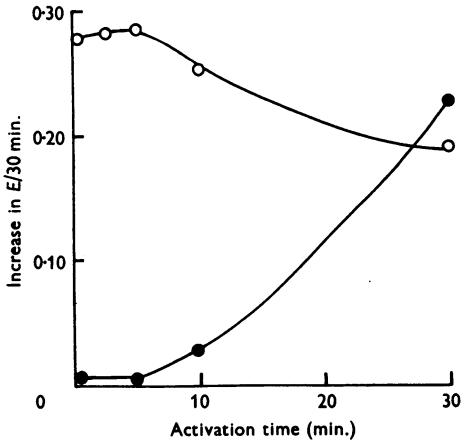

Fig. 4. Activation of human euglobulin. Euglobulin (0·25 ml.) was activated by 60 units of streptokinase (○) or of staphylokinase (●). Conditions were as described for Fig. 2.

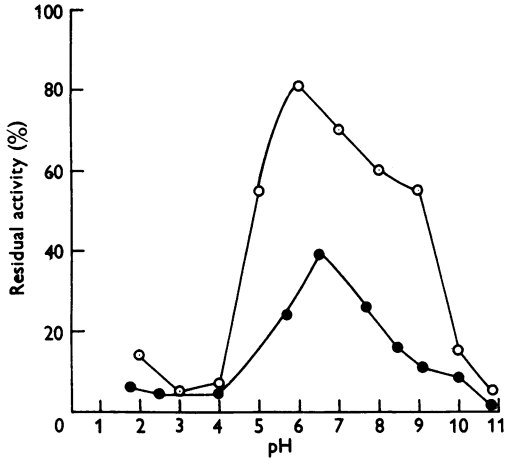

Fig. 5. Heat stability of staphylokinase at different pH values. Samples of crude supernatant (43 units/ml.; ○) and of concentrated staphylokinase (1150 units/ml.; ●) were maintained at 100° for 30 min. The method of assay is described in the Experimental section.

reached depended on the concentration of staphylokinase as well as on the concentration of plasminogen. However, even with a high concentration of plasminogen and a low one of staphylokinase, fresh plasminogen added after 4 hr. was activated at the initial rate (Fig. 3), indicating that the kinase is not used up in the formation of plasmin, but that the reaction is probably enzymic.

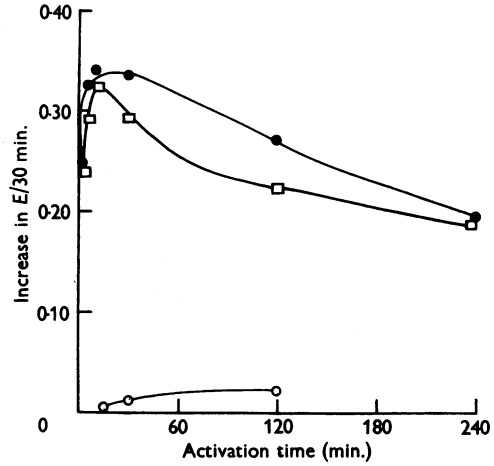

Fig. 6. Activation of rabbit plasminogen. Rabbit euglobulin (0·3 ml.) was incubated for the times shown: ○, with streptokinase (50 units); ●, with staphylokinase (50 units); □, with streptokinase (50 units) plus human plasminogen ($3{\cdot}5 \times 10^{-3}$ unit). A portion (1 ml.) of 4% (w/v) casein was then added and the proteolytic activity determined as described in the Experimental section. With streptokinase plus human plasminogen, the control value obtained in the absence of rabbit euglobulin was deducted.

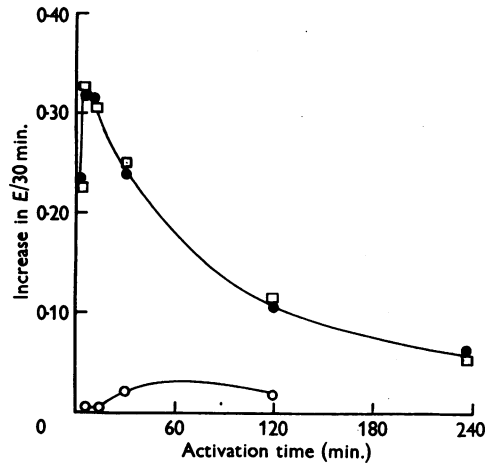

Fig. 7. Activation of guinea-pig plasminogen. Guinea-pig euglobulin (0·2 ml.) was used: ○, with streptokinase (50 units); ●, with staphylokinase (50 units); □, with streptokinase (50 units) plus human plasminogen ($3{\cdot}5 \times 10^{-3}$ unit). Other conditions were as described for Fig. 6.

Inhibition by casein. Activation by staphylokinase was inhibited by casein, or by an impurity in the casein used (Table 1). Because of this, before the addition of casein an activation period of 30 min., with an excess of plasminogen, was used for the assay of staphylokinase, as described in the Experimental section.

Activation of plasminogen in human euglobulin. The plasminogen in human euglobulin was more slowly activated than purified plasminogen. Comparison with streptokinase activation demonstrated the presence of an inhibitor of staphylokinase in the four samples of human euglobulin tested. An example is shown in Fig. 4.

Stability of staphylokinase. The crude supernatant, obtained by removal of organisms from the staphylococcal culture, was stable between pH 2 and 11 for 24 hr. at 5°. At pH 7 it could be heated for 1 hr. at 70° or for 10 min. at 100° without loss of activity. Concentrated staphylokinase was equally stable at 5° but rather less stable at 100°. Maximum stability was at pH 6–7 (Fig. 5). Adjustments of pH were made with 10N-HCl or 10N-NaOH.

Activation of other plasminogens by staphylokinase

Rabbit and guinea-pig plasminogens. Staphylokinase activated the plasminogens in rabbit and guinea-pig euglobulins as shown in Figs. 6 and 7.

Ox plasminogen. Staphylokinase did not activate ox plasminogen. Euglobulin (0·1–0·6 ml., representing 0·25–1·5 ml. of serum) was incubated with staphylokinase (50–1200 units) for various times up to 4 hr. Casein was then added and incubation continued for 30 min.–4 hr. In no case was any activity observed. These negative results were confirmed by testing on heated and unheated ox-fibrin plates (Lassen, 1952). They were not due to an inhibitor of staphylokinase since the activation of human plasminogen (9×10^{-3} unit) by 50 units of staphylokinase was not affected by the addition of ox euglobulin.

Comparison of staphylokinase and streptokinase

Activation of human plasminogen. Various concentrations of staphylokinase and streptokinase could be equated to give approximately the same rate and extent of activation of purified human plasminogen (Figs. 1, 2 and 8). The rates of inactivation of plasmin were also similar.

Streptokinase was slightly inhibited by casein but to a much lesser extent than was staphylokinase (Table 1 and Fig. 9).

Activation of other plasminogens. Streptokinase did not activate rabbit, guinea-pig or ox plasminogens.

'Proactivator' in plasminogen activation

Activation by streptokinase. Rabbit, guinea-pig and ox plasminogens were activated by a mixture of streptokinase and human plasminogen. Rabbit

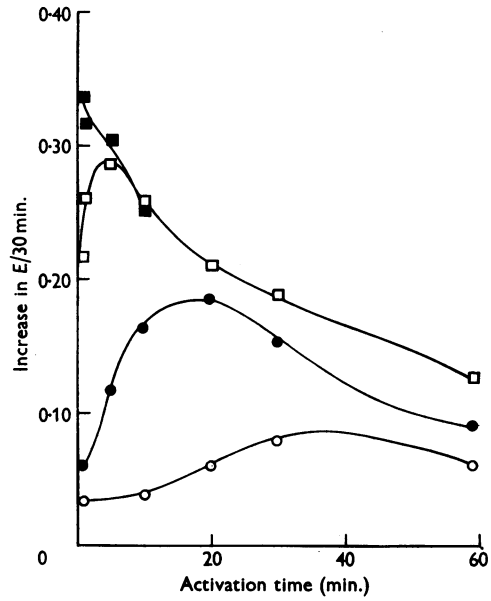

Fig. 8. Activation of purified human plasminogen by streptokinase. Conditions were exactly as described for Fig. 2. Amount of streptokinase: ○, 0·5 unit; ●, 5 units; □, 50 units; ■, 500 units.

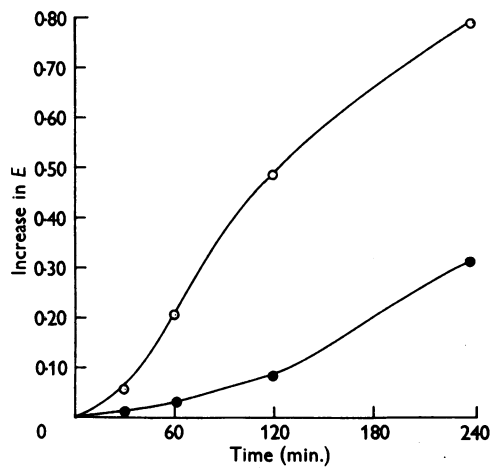

Fig. 9. Activation of plasminogen in the presence of casein. Human plasminogen ($11{\cdot}2 \times 10^{-3}$ unit), streptokinase (○) or staphylokinase (●) (5 units), and 1 ml. of 4% (w/v) casein, in a total volume of 2·1 ml., were incubated for the times shown, without prior incubation of the enzyme system. The total digestion of protein was measured as described in the Experimental section.

and guinea-pig plasminogens gave the same activity with this system as with staphylokinase (Figs. 6, 7).

Activation by staphylokinase. The activation of rabbit and guinea-pig plasminogens by staphylokinase was not affected by the addition of human plasminogen. Nor was ox plasminogen activated by a mixture of staphylokinase and human plasminogen. This was tested under the conditions described for ox euglobulin and staphylokinase, with the addition of $1{\cdot}8 \times 10^{-3}$ unit of human plasminogen.

Species differences in serum plasminogen

A summary of the results with the four species studied is given in Table 2.

DISCUSSION

The species specificity found for staphylokinase was in agreement with previous observations (Gerheim & Ferguson, 1949; Hayashi & Maekawa, 1954). The slow or incomplete activation found with staphylokinase by Lewis & Ferguson (1951), Cliffton & Cannamela (1953) and Hayashi & Maekawa (1954) was probably due to an insufficiently active preparation of staphylokinase or to the presence of inhibitors, e.g. in casein and human globulin. When appropriate concentrations of streptokinase and staphylokinase were compared, the rates and extents of activation of purified human plasminogen were almost identical.

Ox plasminogen was not activated either by staphylokinase alone or by a mixture of staphylokinase and proactivator under a wide range of conditions suitable for the demonstration of its activation by streptokinase and proactivator. This is in agreement with the observations of Hayashi & Maekawa (1954) and of Celander & Guest (1959), made by different methods, and indicates, along with its wider species specificity, that staphylokinase does not act through the proactivator system. Staphylokinase may activate plasminogen directly but it does not closely resemble tissue activator or urokinase, which have been postulated to be direct activators of plasminogen, as they both readily activate ox plasminogen (Astrup, 1956). Though staphylokinase activation of human plasminogen was not inhibited by ox euglobulin, the presence of a highly specific inhibitor for the activation of ox plasminogen has not been entirely excluded. Plasminogens are probably activated by more than one mechanism (Markus, Ambrus, Whistler & Woernley, 1958; Alkjaersig, Fletcher & Sherry, 1959; Olesen, 1959).

By the use of prolonged incubation at 4° to prevent loss of plasmin, Lewis & Ferguson (1951) concluded that the activation of dog plasminogen by staphylokinase was enzymic. The present observation that the activity of staphylokinase did not disappear during the reaction supports the view that the formation of plasmin is catalytic, though it does not rule out the stoicheiometric formation of an intermediate activator, as postulated by Müllertz (1955) for streptokinase.

The values for the plasminogen content of human and guinea-pig sera are, when corrected for the modification of the method, in good agreement with those obtained by Norman (1957).

Table 2. *Activation of plasminogen from four species*

Experimental details are given in the text. The plasminogen content of serum was calculated from the maximum activity obtained from the euglobulin fraction.

	Human	Guinea pig	Rabbit	Ox
Streptokinase	+	−	−	−
Streptokinase + proactivator	+	+	+	+
Staphylokinase	+	+	+	−
Staphylokinase + proactivator	+	+	+	−
$10^3 \times$ Plasminogen (units/ml. of serum)	40	57	34	28

SUMMARY

1. Staphylokinase activated human, rabbit and guinea-pig plasminogens but not ox plasminogen. Unlike streptokinase, its action on animal plasminogens was not affected by the addition of human plasminogen (proactivator).

2. The results obtained support the view that the activation of plasminogen is enzymic. Staphylokinase was not destroyed during the activation of human plasminogen.

3. A caseinolytic method for the assay of kinase activity is described.

4. Crude staphylokinase was stable between pH 2 and 11 at 5°. It withstood heating at 100° for 10 min. at pH 7.

I wish to acknowledge the gift of fraction III of human plasma from E. R. Squibb and Sons, by courtesy of the American National Red Cross. Some of the equipment used in this research was purchased from a grant from the University of London to Dr C. H. Lack, in whose Department this work was done.

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A Method for Determining the Concentration of Ubiquinone in Mitochondrial Preparations

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In the course of studies on the possible role of ubiquinone (coenzyme Q) in the respiratory chain it became necessary to devise a rapid and accurate method for the assay of the substance in small amounts of tissue preparations. The method consists essentially of: (*a*) the simultaneous termination of any enzymic reaction and denaturation of enzyme proteins without destroying the ubiquinone or altering the oxidation–reduction state of ubiquinone–ubiquinol mixtures; (*b*) extraction of the lipid with light petroleum; (*c*) removal of interfering lipids, such as phospholipids, by partitioning the lipid between light petroleum and methanol; (*d*) the spectrophotometric determination of the ubiquinone. The method can be used for the determination of the ubiquinone concentrations in mitochondria or other preparations, except in special cases where the concentration of other substances with u.v.-light absorption (e.g. vitamin A) is high enough to interfere with the determination. It can also be used for kinetic studies where a knowledge of the oxidation–reduction state of the quinone is required (Redfearn & Pumphrey, 1960).

MATERIALS AND METHODS

Mitochondria. These were prepared from animal tissues by the method of Schneider & Hogeboom (1950) and from plant tissues by the method of Simon (1957).

Heart-muscle preparations. These were prepared from pig heart by a modified Keilin & Hartree (1947) procedure. Fresh minced heart muscle (400 g.) was washed with cold water (2 l.) for about 15 min., collected by straining off the water through mutton cloth and squeezed to remove excess of liquid. The washing process was repeated six or seven times until the washings were colourless. The washed mince was homogenized for 1·25 min. in a Waring Blendor with 0·1 M-KH_2PO_4–Na_2HPO_4 buffer, pH 7·4 (400 ml.). The homogenate was centrifuged for 20 min. at 1800 *g* to remove nuclei, unbroken cells and muscle fibres from suspension. The dark-red turbid supernatant containing the enzyme particles (mitochondrial fragments) was decanted and centrifuged at 20 000 *g* for 30 min. The resulting precipitate was divided into two parts: one part was suspended in 0·1 M-phosphate buffer, the other in 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) –HCl buffer, pH 7·4, and both suspensions were centrifuged at 55 000 *g* for 15 min. The precipitates of washed enzyme particles were suspended in the same buffers to give a protein concentration of 10–30 mg./ml. The whole procedure was carried out at 0°.

Solvents. Ethanol was purified for spectrophotometric use by adding zinc dust (20 g.) and potassium hydroxide (40 g.) to ethanol (1 l.), and refluxing the mixture for at least 1 hr. before distilling. Light petroleum (A.R., b.p. 40–60°) was passed through a column (20 cm. × 1·5 cm.) of silica gel (200-mesh) to remove benzene and then distilled. Methanol (A.R.) was redistilled.

Protein determinations. These were made by the biuret method (Cleland & Slater, 1953), purified fat-free casein being used as the standard.

Assay of ubiquinone. A portion (1 ml.) of the tissue preparation (mitochondria or heart-muscle preparation) containing 10–30 mg. of protein was placed in a 15 ml. conical centrifuge tube. The preparation was denatured by the rapid addition from a hypodermic or spring-loaded pipetting syringe of cold (−20°) methanol (4 ml.)