2. The liver, liver tumour, kidney and spleen were homogenized and soluble extracts of the tissues were obtained. Samples of the extracts were treated with trichloroacetic acid and the radioactivity of the lysine and alanine in hydrolysates of the precipitated proteins was determined. The radioactivity of the alanine and lysine occurring free in the supernatants of the trichloroacetic acid precipitates was also determined.

3. The amount of serum albumin in the tissue extracts was determined by means of an antiserum. From estimations of the amount of blood in the extracts it could be shown that the total amount of albumin in the extracts greatly exceeded that due to the presence of blood.

4. The plasma proteins obtained from the rat at death were fractionated by zone electrophoresis. Fractions containing albumin plus α-globulin, β-globulin and γ-globulin were obtained. The radioactivity of the alanine and lysine in hydrolysates of each fraction was determined.

5. From the ratio of the radioactivity of the free amino acids in the tissues to that of the amino acids in the tissue proteins it is concluded that there is no evidence that serum albumin is utilized for the synthesis of soluble tissue proteins without first being broken down to free amino acids. In this respect liver tumour did not appear to differ from the other tissues studied.

6. No evidence could be found for the interconversion of serum albumin and serum globulin at other than an amino acid level.

We are grateful to Professor F. Dickens, F.R.S., for his encouragement, Dr I. M. Roitt for many helpful discussions, Mr H. E. H. Jones for carrying out the laparotomies and the intravenous injections, Dr A. C. Thackray for the histological examinations and Mr J. K. Whitehead for gas counting. A grant to the Medical School by the British Empire Cancer Campaign covered the cost of the work. Finally we should like to thank Miss B. Kernot for skilled technical assistance throughout the course of the work.

REFERENCES

Åkerfeldt, S. (1954). *Ark. Kemi*, **7**, 355.
Askonas, B. A., Campbell, P. N., Godin, C. & Work. T. S. (1955). *Biochem. J.* **61**, 105.
Askonas, B. A. & White, R. G. (1956). *Brit. J. exp. Path.* **37**, 61.
Babson, A. L. & Winnick, T. (1954). *Cancer Res.* **14**, 606.
Busch, H. & Greene, H. S. N. (1955). *Yale J. Biol. Med.* **27**, 339.
Campbell, P. N. (1955). *Biochem. J.* **61**, 496.
Campbell, P. N. (1956). *Biochim. biophys. Acta*, **21**, 167.
Campbell, P. N. & Halliday, J. W. (1957). *Biochem. J.* **65**, 28.
Campbell, P. N., Jones, H. E. H. & Stone, N. E. (1956). *Nature, Lond.*, **177**, 138.
Campbell, P. N. & Stone, N. E. (1957). *Biochem. J.* **66**, 19.
Cocking, E. C. & Yemm, E. W. (1954). *Biochem. J.* **58**, xii.
Coons, A. H., Leduc, E. H. & Kaplan, M. H. (1951). *J. exp. Med.* **93**, 173.
Flynn, F. V. & de Mayo, P. (1951). *Lancet*, **2**, 235.
Godin, C. & Work, T. S. (1956). *Biochem. J.* **63**, 69.
Grabar, P. & Williams, C. A. jun. (1955). *Biochim. biophys. Acta*, **17**, 67.
Heimberg, M. & Velick, S. F. (1954). *J. biol. Chem.* **208**, 725.
Hirs, C. H. W., Moore, S. & Stein, W. H. (1954). *J. Amer. chem. Soc.* **76**, 6063.
Holzer, H., Sedlmayr, G. & Kiese, M. (1956). *Biochem. Z.* **328**, 176.
Humphrey, J. H., Neuberger, A. & Perkins, D. J. (1956). *Biochem. J.* **64**, 2P.
Korner, A. & Debro, J. R. (1956). *Nature, Lond.*, **178**, 1067.
Loftfield, R. B. & Harris, A. (1956). *J. biol. Chem.* **219**, 151.
Maurer, W. & Müller, E. R. (1955). *Biochem. Z.* **326**, 474.
Miller, L. L. & Bale, W. F. (1954). *J. exp. Med.* **99**, 125.
Miller, L. L., Bly, C. G. & Bale, W. F. (1954). *J. exp. Med.* **99**, 133.
Miller, L. L., Bly, C. G., Watson, M. L. & Bale, W. F. (1951). *J. exp. Med.* **94**, 431.
Salmony, D. & Whitehead, J. K. (1954). *Biochem. J.* **58**, 408.
Simpson, M. V. & Velick, S. F. (1954). *J. biol. Chem.* **208**, 61.
Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
Yuile, C. L., Lamson, B. G., Miller, L. L. & Whipple, G. H. (1951). *J. exp. Med.* **93**, 539.
Zamecnik, P. C., Loftfield, R. B., Stephenson, M. L. & Steele, J. M. (1951). *Cancer Res.* **11**, 592.

The Isolation and Properties of a Fragment of Bovine-Serum Albumin which Retains the Ability to Combine with Rabbit Antiserum

By R. R. PORTER
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 18 *January* 1957)

The structural basis of the biological activity of proteins is a problem which is arousing growing interest as knowledge of protein structure increases. One approach (Porter, 1953) is to hydrolyse a protein under conditions in which the biological activity survives and then isolate the fragment responsible for activity, thus reducing the structural work necessary to pinpoint the features essential for activity. Difficulty has always been met in isolating the active fragment, but retention of activity after partial hydrolysis has been demonstrated in a sufficient number of cases to establish that the whole protein molecule is not necessarily essential for activity.

Landsteiner (1942) made such an attempt to find the antigenic combining sites of silk fibroin, and was able to obtain from a sulphuric acid digest fragments which would inhibit the combination of silk fibroin with antisera prepared by injection into a rabbit of the soluble fibroin adsorbed on charcoal. The silk fibroin was brought into solution by heating in acid. It was not certain how far these results could be applied to a native globular protein, but several workers (Landsteiner & Chase, 1933; Holiday, 1939; Kleczkowski, 1945) have reported that serum proteins will retain the power to precipitate with antisera after short periods of enzymic digestion. Recently Lapresle (1955*a*, *b*) has reported that human-serum albumin, if digested with a crude extract of spleen, gives three components which retain the ability to precipitate with rabbit anti-human-serum albumin. These fractions have, at pH 8·6, an electrophoretic mobility which is close to that of the undigested proteins, and it was suggested that they arise from only slight breakdown of the albumin; they were not characterized further.

An attempt has therefore been made to isolate a small fragment of a protein antigen which would retain the power to combine with its antisera. From Landsteiner's work it seemed probable that fragments of the antigenic protein might retain their power to combine specifically with antibody even though visible precipitates were not formed. In this case a sensitive test for the existence of active fragments of antigen would consist in measuring the amount of precipitate formed when antibody and antigen were mixed in optimum proportions in the presence of partly digested antigen. Ovalbumin was first tried as it is a good antigen of relatively low molecular weight, but under none of the conditions used was there any evidence of production of an active fragment which would diffuse through a cellophan membrane. Bovine-serum albumin was then tested and it was found that chymotryptic digestion under appropriate conditions produced diffusible material which was immunologically active both *in vitro* and *in vivo*. This material will be referred to as the inhibitor.

EXPERIMENTAL

Materials

Crystalline bovine-serum albumin was purchased from Armour and Co. Ltd. Goat-serum albumin was prepared by zone electrophoresis. Trypsin and chymotrypsin, recrystallized six times (Northrop, Kunitz & Herriott, 1948), were given by Mr J. Lightbown. Crystalline pepsin was purchased from Armour and Co. Ltd. Crystalline mould protease (Crewther & Lennox, 1950) was given by Dr Crewther. Crystalline papain (Kimmel & Smith, 1954) was given by Dr Emil Smith. Crystalline streptococcal protease (Elliot, 1950) was given by Dr J. Humphrey.

Anti-bovine-serum albumin was prepared by injection into rabbits of the protein precipitated with alum, as described for the preparation of anti-ovalbumin (Porter, 1955). Six rabbits were used and sera are referred to by the rabbit number (i.e. 1–6). Cellophan membranes were purchased from the Visking Corporation, Chicago.

Methods

For zone electrophoresis, cotton wool treated with acid ethanol was used as the supporting solid according to the methods described by Flodin & Kupke (1956) and Porath (1956). Electrophoresis was in 0·025 M-sodium borate, pH 9·4, or in 0·5 N-acetic acid.

N-Terminal amino acids were estimated according to published methods (Porter, 1957) with fluoro-2:4-dinitrobenzene.

The effect of heat denaturation was tested by immersing a solution (1 mg./ml.) of inhibitor in 0·05 M-sodium phosphate, pH 7, in boiling water.

Estimation of inhibitory power. Estimations of inhibitory power of an enzymic digest were made by two methods. An approximate method was used in preliminary investigations and to follow the purification of the active fragment. This was based on the delay in flocculation which occurred when increasing volumes of the digest solution were added to the antisera before addition of the optimum amount of bovine-serum albumin. For example, with one antiserum (16 mg. of antibody/ml.), 1 ml. of 1/50 dilution in 0·15% NaCl was run into a flocculation tube in a bath at 37°. A volume (0·01–0·5 ml.) of test solution was added and mixed; 5 min. later 0·04 ml. of bovine-serum albumin (1 mg./ml.) was added and the tubes were again mixed. The time of flocculation was compared with that of a control tube in which saline had been added to the antiserum in the same volume as that of the inhibitor. The test solution was always brought to pH 7 before testing. A calibration curve was prepared of time of flocculation against volume of an arbitrary standard solution of inhibitor; this gives a rapid semiquantitative method of estimation.

A more exact estimation of the ability of the inhibitor to combine with anti-bovine-serum albumin was made by measuring the weight of specific precipitate formed by adding the optimum amount of serum albumin to antisera after prior addition of increasing amounts of inhibitor. In this case, antisera were diluted with inert serum or saline until their antibody content was about 2 mg./ml. Inhibitor was added to 0·5 ml. of diluted antisera in increasing amounts, and the volumes in all tubes were equalized by the addition of saline. The tubes were stood at 37° for 1 hr. and at 2° overnight. If any precipitate formed, the tubes were centrifuged at 2° and the precipitate was washed three times with 0·5 ml. of ice-cold saline. The supernatant and first washing were combined and stood at 37°. Bovine-serum albumin sufficient to give maximum precipitation in the absence of inhibitor (in this case 0·13 mg.) was added, and the solutions were mixed and stood at 37° for 1 hr. and overnight at 2°. The precipitates were washed as before and both were estimated by dissolving in 3 ml. of 0·1 N-NaOH and reading the absorption in a spectrophotometer at 280 mμ. The values obtained were converted into weight of antibody from previous calibration with rabbit γ-globulin, after allowance for the antigen content of the precipitate. A solution of rabbit γ-globulin in 0·1 N-NaOH which gives a reading of 1·0 at 280 mμ contains 0·715 mg. of protein.

Enzymic hydrolysis of serum albumin. Enzymic digestion of bovine-serum albumin was carried out in a dialysis sac suspended in 10–20 vol. of water which had been adjusted to the same pH as the digestion mixture. The outer liquor (diffusate) was tested for inhibitory activity at regular time intervals. This method was adopted because active material with a considerably reduced molecular size was looked for, and it also offered a chance of obtaining intermediary digestion products which would lose activity if enzymic digestion went to completion. For preliminary work, the solutions were kept at 37° and were shaken continuously to facilitate diffusion.

With trypsin, chymotrypsin, papain and mould protease the solutions were adjusted to pH 8 with aq. 2N-NH_3 soln., and with pepsin were reduced to pH 5 with N-acetic acid or pH 2 with N-HCl. The papain, received as the inactive mercury–enzyme, was activated by cysteine and ethylenediaminetetra-acetic acid (EDTA) as described by Kimmel & Smith (1954) and these compounds were added to the digestion mixture and outer liquid to prevent subsequent inactivation of the enzyme.

Other methods of measurement of antibody–antigen reaction. Antibody–antigen reactions in agar plates were carried out according to the method of Ouchterlony (1953), except that the plates were kept at 2°, not 37°.

Passive anaphylactic shock tests were performed by Dr J. H. Humphrey as follows: six guinea pigs were injected intraperitoneally with 2 ml. of anti-bovine-serum albumin (8·5 mg. of antibody/ml.). Two days later three were challenged by intravenous injection of bovine-serum albumin in decreasing amounts (2, 0·5 and 0·05 mg.). The other three were injected with the same amounts of inhibitor.

RESULTS

When ovalbumin was hydrolysed by the six proteases under the conditions given above, no precipitating or inhibitory activity was detectable in the diffusate.

When bovine-serum albumin was hydrolysed similarly, activity was found in the diffusate if trypsin or chymotrypsin was the digesting enzyme. No activity was found when papain, pepsin, mould protease or streptococcal protease was used.

The activity of the diffusate was at least five times greater when chymotrypsin was used than with trypsin. The work with trypsin has not been pursued further, and in the work described here chymotryptic digestion has been used throughout. If the bovine-serum albumin and chymotrypsin are incubated separately in dialysis sacs in an identical manner with that used for digestion, no activity is detectable in the diffusate. It was therefore concluded that the activity which dialysed through the cellophan must arise from a product of the enzymic digestion and conditions were found which would give a high yield for preparative purposes.

Further incubation of the inhibitor solution with chymotrypsin in a glass vessel destroyed its activity. It therefore appeared that we were dealing with an intermediary product of hydrolysis, which had at least one essential bond susceptible to chymotrypsin but more resistant than other bonds broken during release of this material from the whole protein. Conditions were therefore changed to reduce the rate of hydrolysis relative to the rate of diffusion. This was done by working at 25° and reducing the amount of enzyme relative to substrate. A significant rise in inhibitory activity resulted. The conditions finally chosen were as follows: Bovine-serum albumin (2 g.) and 15 mg. of chymotrypsin were dissolved in 5 ml. of water and aq. 2N-NH_3 soln. was added to bring the pH to 8. One drop of toluene was added and the mixture put into 8/32 Visking dialysis sac. The sac was washed very thoroughly outside and then put into 100 ml. of water, adjusted to pH 8 and saturated with toluene in a 100 ml. glass-stoppered measuring cylinder and shaken continuously at 25°. At 3, 6, 12, 24, 30, 36 and 48 hr., and subsequently daily, the outer liquid was changed. A worth-while yield of inhibitor was obtained up to 14 days when the reaction was stopped. At this point, the material remaining in the cellophan bag (the dialysate) retained the power to precipitate with bovine-serum albumin. It has not been investigated further. The scheme of purification of the active material in the diffusate is summarized in Scheme 1.

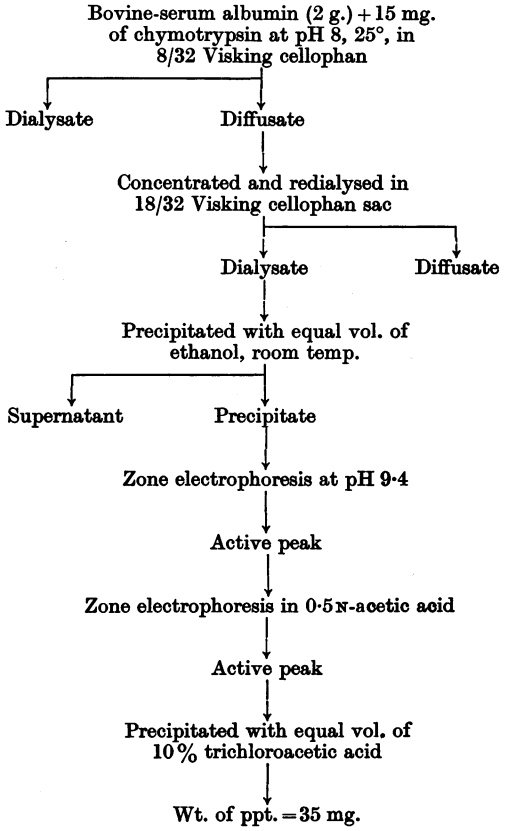
The diffusate was combined into three batches and was concentrated by evaporation in a rotary still with the bath at 30°; 1200 ml. of diffusate was reduced to about 15 ml. in 12 hr.

When different batches of cellophan were examined, an 18/32 Visking sac was found which would completely retain inhibitory activity. Wide variation in the permeability of unstretched cellophan sacs is known to occur (cf. Craig & King, 1955, 1956); advantage was taken of the finding that the inhibitor would pass through one type of sac but not through another, to purify the material further. The concentrated diffusate was dialysed in the 18/32 sac in a rocking dialyser with four changes of water. About half the weight was lost without loss of activity. Though big differences in the permeability of different batches of commercial cellophan occur, it is rare to find any which retains material with a molecular weight below 5000 or allows material with a molecular weight over 20 000 to diffuse through. It therefore seemed probable that the molecular weight of the inhibitor would fall somewhere in this range.

An equal volume of ethanol was added to this second dialysate at room temperature and the precipitate centrifuged after standing for 1 hr.; 90% or more of the activity was contained in the precipitate, which was taken up in 3 ml. of water and after being centrifuged to remove traces of insoluble substance, was fractionated by electrophoresis in a column of treated cotton wool (Porath,

1956). After 30 hr. the material was eluted from the column and an elution diagram is shown in Fig. 1. There was a variety of components, as judged by absorption at 280 mμ, but the activity was confined to the slowest-moving fraction. This fraction was concentrated in a rotary still, dialysed and again fractionated by electrophoresis in 0·5 N-acetic acid in a column. Fig. 2 shows the elution diagram, which at this stage shows a single peak, and if the active fraction is rerun a symmetrical peak is obtained. However, *N*-terminal amino acid assay with fluorodinitrobenzene showed that there were several minor components in addition to the main terminal amino acid. If the material from the acetic acid electrophoresis was precipitated in 5% trichloroacetic acid the precipitate dissolved readily in 0·05 M-phosphate, pH 7, and there was no loss of activity. There was also very little loss of weight, but when *N*-terminal amino acid assay was repeated only phenylalanine was found and all trace of other amino acids had gone. They presumably arose from small amounts of adsorbed peptides which remained in solution during precipitation with trichloroacetic acid.

Scheme 1. Scheme of purification of inhibitor



Properties of the inhibitor

Electrophoresis. As zone electrophoresis at pH 9·4 and 3·5 had been used to purify the inhibitor, it was to be expected that it would appear to be homogeneous by this criterion. The inhibitor was completely adsorbed on treated cellulose near neutrality but gave a single symmetrical peak if examined in phosphate buffer at pH 7 by free

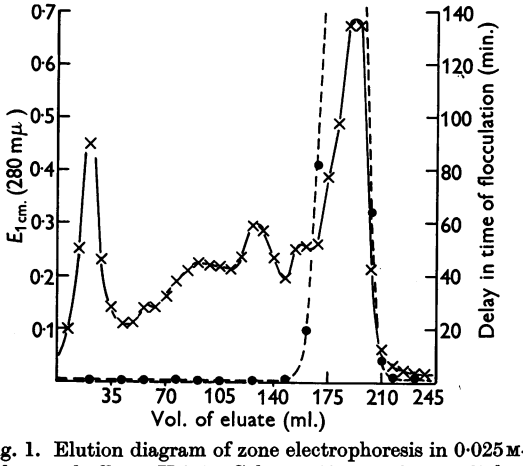


Fig. 1. Elution diagram of zone electrophoresis in 0·025 M-borate buffer, pH 9·4. Column 40 cm. × 3 cm.; 27 hr., 300 v, 40 mA. ——, Extinction coefficient at 280 mμ; – – –, delay caused in time of flocculation on addition of sample of eluate (0·1 ml.) to bovine-serum albumin and rabbit-antiserum system. Time of control flocculation without any addition, 3 min.

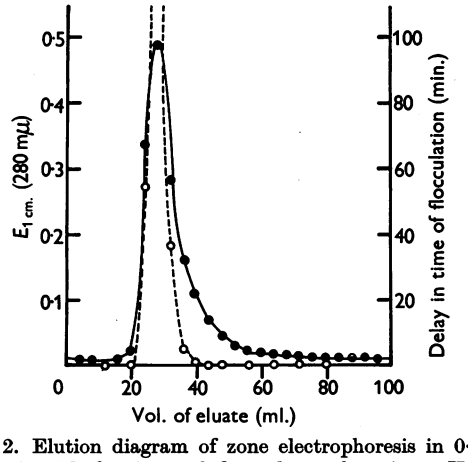


Fig. 2. Elution diagram of zone electrophoresis in 0·5 N-acetic acid of active peak from electrophoresis at pH 9·4. Column 42 cm. × 2 cm.; 7 hr., 550 v, 20 mA. ——, Extinction coefficient at pH 9·4; – – –, delay caused in time of flocculation on addition of sample of eluate (0·1 ml.) to bovine-serum albumin and rabbit-antiserum system. Time of control flocculation without any addition, 3½ min.

electrophoresis or by zone electrophoresis at pH 9·4 and 3·5.

N-*Terminal amino acid.* The fluorodinitrobenzene method showed that phenylalanine was the *N*-terminal acid. After correction for loss during hydrolysis and chromatography the yield was 1 mole/14 000 mol.wt. No traces of other *N*-terminal acids were detectable.

Molecular weight. Dr P. A. Charlwood examined the material in the ultracentrifuge and found it to be monodisperse, judged by the facts that it gave a single symmetrical peak and that there was no change of molecular weight with time at the meniscus in spite of considerable change of concentration. In 0·15M-NaCl–0·05M-potassium phosphate buffer, pH 7·1, the sedimentation constant corrected to water at 20° was found to be 2·13*S* at 0·7% concentration. In 0·05M-borate buffer, pH 9·4, it was 2·29*S* at 0·5% concentration. Estimation of molecular weight was made in each case by Archibald's method (Archibald, 1947; Charlwood, 1957) and showed some difference. In phosphate buffer the molecular weight was found to be 27 000 and in borate buffer 24 000. The reason for this discrepancy is not known.

This molecular weight was about double that expected from *N*-terminal acid assay and was higher than expected for a compound which would pass through a cellophan membrane. The ability of the inhibitor to diffuse through cellophan was therefore retested, and it was found that no activity was detectable in the diffusate when dialysis was carried out under the identical conditions at 25° and pH 8 used for digestion. It appeared that the molecular weight had changed during purification. With another preparation of inhibitor the ability to diffuse through the 8/32 membrane was tested after each step. This remained after concentration of the diffusates and after the second dialysis in 18/32 cellophan tubing. It remained after ethanol precipitation but, if this material was freeze-dried, then the power to diffuse was completely lost. If not freeze-dried but submitted to electrophoresis directly at pH 9·4 the power to diffuse was again lost.

As this change was not necessarily associated with purification, it was assumed that some kind of association had occurred, and conditions were sought under which it could be reversed. Ability to diffuse was used to test for such a reversal. The purified inhibitor was dialysed against 0·05M-Na_2CO_3 and 0·01N-HCl, i.e. at pH 10·5 and 2. There was no loss of inhibitory power under these conditions. No activity came through the sac and it therefore appeared unlikely that electrostatic bonds were involved in the association.

The inhibitor was dialysed against different concentrations of urea at pH 7. There was a slow loss of activity when 1·5M-urea was used and about half the activity was lost in 3M-urea over 24 hr. at room temperature, but again no activity appeared in the diffusate. The involvement of hydrogen bonding in the association therefore seemed improbable.

On the assumption that the association might have arisen from the formation of intermolecular S–S bonds, attempts were made to dialyse the diffusate in a reducing medium. In neutral M-thioglycollate the inhibitor solution gelled and all activity was lost in about 30 min. at room temperature. Intramolecular disulphide bonds in the molecule are probably essential for its activity. In neutral 0·05M-thioglycollate there was slow loss of activity and no diffusion of active material. In neutral 0·02M-cysteine little if any activity was lost but some appeared to diffuse. In acid solution, 0·1M-acetate (pH 4)–0·001M-EDTA–0·02M-cysteine, the inhibitor diffused through the cellophan without loss of activity and when the sedimentation constant was measured under these conditions it showed a substantial reduction. If the solution is neutralized, the power to diffuse is again rapidly lost.

In the acetate buffer the sedimentation constant corrected to water at 20° was 1·42*S* at 0·7% concentration. When the molecular weight was calculated as before, it was found to be 12 000.

It appears that the true molecular weight of the fragment released from bovine-serum albumin is 12 000–13 000 and that it dimerizes through interaction of sulphydryl groups. Crystalline preparations of bovine-serum albumin contain 0·7–0·8 sulphydryl group/molecule (Hughes, 1950). No interaction occurs except through a bivalent heavy metal, such as mercury, when a dimer forms. A bivalent organic mercurial causes the dimer to form much more rapidly (Straessle, 1951). It has been suggested that 70–80% of the molecules have a single sulphydryl group which is prevented from interaction by steric factors. It would appear possible that this single sulphydryl group is present in the inhibitor fragment but that loss of other parts of the serum-albumin structure now enabled it to interact with the same group in another molecule, in the absence of heavy metals. The monomer is stabilized in some way in the impure state but it dimerizes very rapidly when pure, particularly in conditions suitable for oxidation, such as neutral or alkaline pH.

A comparison was made of the immunological behaviour of the inhibitor when in the form of a monomer and a dimer. This was not easy as dimerization occurs rapidly in neutral solution in the absence of cysteine, and moreover, since incubation of antibody with a reducing agent delays its precipitation with the antigen (Porter, 1950*a*), it was

undesirable to work in the presence of cysteine. The behaviour of a solution of the ethanol precipitate before and after freeze-drying was therefore compared. A solution, of which the activity would diffuse readily through cellophan, was divided into two parts. One was freeze-dried and then redissolved in the original volume of water and found to contain no diffusible activity. The inhibitory power of this solution was now compared with that of the original. No qualitative or quantitative difference could be found and it is concluded that the dimerization, which must result in the blocking of a considerable part of the surface of the molecule, has not influenced its power to combine with antibody. All the immunological work described later was carried out with the purified inhibitor, i.e. the dimer.

Amino acid analysis. Preliminary amino acid analysis was made by Dr S. Jacobs using a procedure similar to that of Moore & Stein (1951). Table 1 shows the results obtained and they are compared with the values reported for bovine-serum albumin (Stein & Moore, 1949). As only one estimation was made with the inhibitor, no high degree of accuracy can be claimed, but it can be seen that there are some striking differences between the amino acid content of this fragment and the whole molecule. As the molecular weight of the inhibitor is about 12000 and represents about a fifth part of the original molecule, it is clear that there must be considerable differences in the distribution of amino acids along the peptide chain of serum albumin.

The cystine analysis gives a figure of 1·4 moles/12 000, suggesting that there is one disulphide bond and a free sulphydryl group. Experiments described above to determine the molecular weight suggest that rupture of the disulphide bond destroys activity and that the free sulphydryl group is responsible for the dimerization which occurs.

Stability. The inhibitor shows varying stability in different conditions. The solubility and activity are unaffected by precipitation in ethanol at room temperature, by precipitation with 5% trichloroacetic acid, by exposure to pH 10·5 or pH 2 over 24 hr. at room temperature. Solubility and activity are lost by heating to 100° for 2–3 min. or by standing in M-thioglycollate at pH 7. Activity is also destroyed slowly by comparatively low concentrations (1·5M) of urea.

The native configuration of the peptide chain, controlled by both a disulphide bond and hydrogen bonds, must be necessary for the inhibitor to be able to combine with anti-bovine-serum albumin.

Immunological properties of the inhibitor

The time taken for the inhibitor to react with the antisera was estimated approximately by mixing antisera and inhibitor and adding bovine-serum albumin after varying times. After incubation the weight of specific precipitate formed was estimated.

The results (Table 2) indicate that inhibitor, present in excess, reacted completely with antibody in less than 2 min. but that when antigen and inhibitor reacted simultaneously more precipitate formed. If the combination of inhibitor and antigen with antibody were easily reversible reactions the same weight of precipitate would have been expected in all cases.

In order to eliminate the effect of time of reaction at least 5 min. was always allowed in subsequent experiments between addition of inhibitor and serum albumin to the antisera.

Table 1. *Amino acid analysis of inhibitor and of serum albumin*

Results are expressed as g./100 g. of protein.

Amino acid	Inhibitor	Serum albumin*
Aspartic acid	9·0	10·9
Threonine	7·2	5·8
Serine	2·0	4·2
Glutamic acid	16·3	16·5
Proline	7·3	4·8
Glycine	1·9	1·8
Cystine	2·7	6·0
Alanine	5·6	6·3
Valine	9·3	5·9
Methionine	0·8	0·8
Leucine	11·6	12·3
Isoleucine	2·8	2·6
Tyrosine	1·2	5·1
Phenylalanine	6·1	6·6
Tryptophan	0†	0·58
Lysine	12·9	12·8
Histidine	2·1	4·0
Arginine	5·4	5·9

* The amino acid content of serum albumin has been taken from Stein & Moore (1949).

† Tryptophan could not be detected spectroscopically with the Holiday photographic method (Beaven & Holiday, 1952).

Table 2. *Effect of times of addition of inhibitor and antigen to antiserum on the weight of precipitate formed*

To 0·5 ml. of serum (2 mg. of antibody/ml.) was added excess of inhibitor (0·2 mg.). This was followed by 0·14 mg. of bovine-serum albumin after different time intervals. The first tube (zero time) was mixed after rapid addition of serum albumin, the others after addition of the inhibitor, and serum albumin was added after 2 min., 10 min. and 48 hr., and was again mixed. The solutions were subsequently stood at 37° for 2 hr. and at 2° for 48 hr.

Time of addition of serum albumin after mixing inhibitor and antiserum	Wt. of precipitate formed (mg.)
0 min.	0·63
2 min.	0·48
10 min.	0·47
48 hr.	0·47

Effect of additon of inhibitor to different anti-bovine-serum-albumin sera

Increasing amounts of inhibitor were added to a constant amount of antiserum in absence of serum albumin and any precipitate formed was estimated as described. The amount of antibody remaining in the supernatant and still able to form a precipitate with bovine-serum albumin was also estimated. This experiment was repeated with goat-serum albumin instead of bovine-serum albumin. This enabled us to decide how the antibodies with which the inhibitor was combining were related to the antibodies which would cross-react with the heterologous goat-serum albumin.

It quickly became clear that the results obtained varied considerably from one serum to the next. No two antisera behaved the same, whether obtained from different rabbits after the same course of injections or from the same rabbit after successive courses of injections.

Fig. 3 shows the results with rabbit 1 after six injections given in 2 weeks. With this serum the inhibitor alone gave a precipitate which at the maximum was about one-quarter of the precipitate given by bovine-serum albumin in optimum proportions. The combining ratio of inhibitor to antibody was 1:8·5, very similar to that found with bovine-serum albumin. This was the only serum tested in which sufficient precipitate was formed on addition of inhibitor alone for satisfactory estimation. The precipitate formed by addition of serum albumin to the supernatant fell in the presence of increasing amounts of inhibitor to a minimum of about two-thirds of that formed in the absence of inhibitor. When goat-serum albumin was used to precipitate the antibody from the supernatant, only one-quarter was precipitated in the absence of inhibitor and one-eighth in the presence of excess of inhibitor. Of the antibodies which precipitated with goat-serum albumin about one-half are able to combine with the inhibitor also.

When serum from the same rabbit, after a further six injections of bovine-serum albumin, was tested quite different results were obtained. There was a slight precipitation with the inhibitor alone but it was too small to measure satisfactorily and may have been non-specific. As is shown in Fig. 4 the inhibitor was, however, apparently able to combine with all the antibody and there was complete inhibition of precipitation with either bovine- or goat-serum albumin. If the straight-line portions of the graphs are extrapolated to complete inhibition, 0·8 mg. of inhibitor is found to be required to inhibit 2·2 mg. of bovine-serum albumin. If the molecular weight of inhibitor is 12 000 and of bovine-serum albumin 66 000, the molar ratio of inhibitor:serum albumin at complete inhibition is 1·8:1.

Reaction of inhibitor and antisera in agar gel

It was observed that the inhibitor would form a visible opaque zone in agar gel when allowed to diffuse towards an antiserum with which it would not form a precipitate in free solution. Aggregates are apparently formed, which cause visible opacity in the gel.

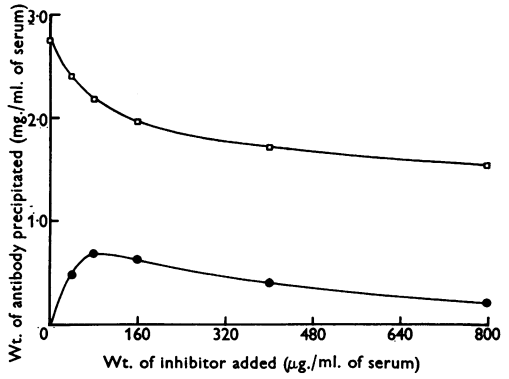


Fig. 3. Amount of precipitate formed on addition of increasing weights of inhibitor and serum albumin to 1 ml. of rabbit anti-bovine-serum albumin, serum 1*a*. ●, Precipitate formed on addition of inhibitor alone; □, precipitate formed on addition of constant weight of bovine-serum albumin to supernatant from inhibitor precipitate.

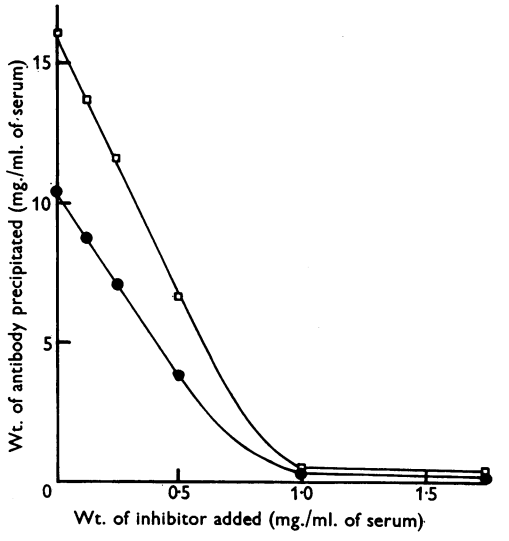


Fig. 4. Amount of precipitate formed on addition of constant weight of serum albumin, in the presence of increasing weight of inhibitor, to rabbit anti-bovine-serum albumin, serum 1*b*. □, Precipitate formed on addition of bovine-serum albumin; ●, precipitate formed on addition of goat-serum albumin.

The behaviour of the inhibitor was compared with that of bovine-serum albumin by the Ouchterlony (1953) technique. Pl. 1 shows the results obtained when serum albumin and inhibitor were tested at three concentrations: 5, 0·5 and 0·05 mg./ml. against undiluted serum 3 (8 mg. of antibody/ml.). At the lowest concentration, hardly any opaque zone was detectable, but at the middle and higher concentrations a Y type of interaction can be seen which is very similar to that obtained when a comparison is made, by this method, of homologous and heterologous antigens (Ouchterlony, 1953). Presumably a similar explanation holds. When serum albumin and antisera meet, all the antibodies are precipitated. When inhibitor and antisera meet, only a proportion of the antibodies precipitate; some antibody unable to combine with inhibitor continues to diffuse until it meets the bovine-serum albumin front and precipitates there. This is the explanation offered for the behaviour of, say, bovine- and goat-serum albumin set up in the same way, but in our experiment we have the whole and part of the same protein, not two related proteins.

Reaction of the inhibitor in vivo

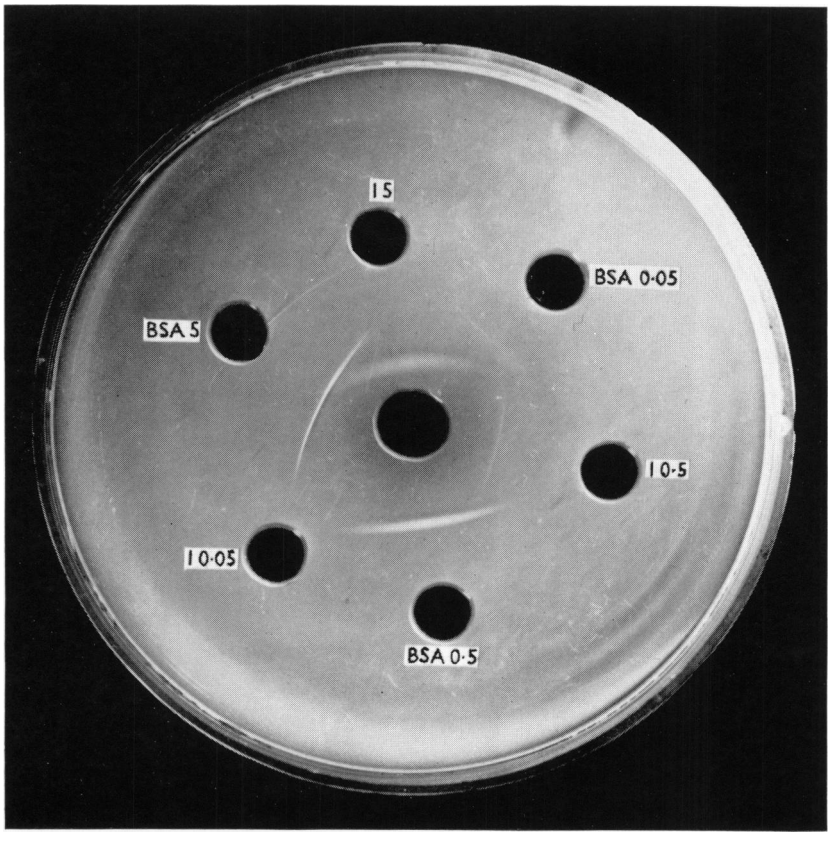
The power of the inhibitor to provoke anaphylactic shock in a guinea pig, which had been passively immunized 2 days earlier with rabbit anti-bovine-serum albumin, was tested by Dr H. J. Humphrey. The guinea pigs were challenged by intravenous injection of bovine-serum albumin or inhibitor at three concentrations, and it was found that shock occurred with either substance and at the same concentration levels, thus demonstrating that reaction with inhibitor had the same biological effect as with the whole molecule.

DISCUSSION

The experimental results suggest that the active fragment formed by chymotryptic digestion of bovine-serum albumin has been obtained pure. It appears to be homogeneous by electrophoresis and also in the ultracentrifuge, both as the monomer and the dimer. Only one *N*-terminal amino acid was found and it was present in approximately molar ratio, taking the molecular weight estimated by ultracentrifuge as 12 000. It therefore seems probable that the effects observed are a property of this fragment with a molecular weight of 12 000 and are not due to any contaminant. The recovery of activity was tested at each stage and there was very little loss of activity in any step. It is unlikely therefore that any other active fragment was produced under the conditions of hydrolysis that have been used. There was, however, evidence that an active component moving faster on electrophoresis at pH 9·4 was formed when the digestion was carried out at 37°. There was very little of this second active component, and it was not of significantly smaller size as it also would not diffuse through the 18/32 cellophan sac. Similarly, the active product of tryptic digestion would not dialyse through the 18/32 cellophan. When tested together there was no evidence to suggest that the three inhibitors from bovine-serum albumin had an independent and additive inhibitory power. Owing to the complexity of the antibodies in any antiserum interpretation is uncertain, but it would appear to mean that each inhibitor contained the same combining site or sites. This may be a reflexion on the amino acid distribution along the peptide chain which makes this section relatively resistant to certain enzymes and has caused essentially the same part of the antigen to be obtained in each case. Loss of activity by heating, or reduction of the disulphide bond, shows that the configuration is still an essential feature and, if this is generally true of the antigenic combining sites of native proteins, it explains the difficulty in producing small active fragments. Configuration at any point is likely to be controlled by a relatively large adjoining section of the protein. Failure to detect any change in inhibitory power when the monomer dimerizes shows that a large part of the inhibitor adjoining the free sulphydryl group must not take part in the combination with antibody as it will be blocked in the dimer. This section containing the sulphydryl group may well, however, contribute to the control of the configuration of the active site. Attempts to reduce the size of the inhibitor further, without loss of activity, have so far been unsuccessful, but negative results have little significance in this type of experiment.

The finding that a short but detectable time is required between addition of inhibitor and antigen to achieve full inhibition, and that this is not reversed even after the reactants are allowed to remain together for long periods, suggests that the combination of inhibitor and antibody is irreversible or very slowly reversible. This is in agreement with the finding that full inhibition can be obtained with a molar ratio of inhibitor to antigen of as low as 1·8:1. If the inhibition were competitive then a higher ratio would be expected and the same final result from mixing the same proportions of reactants should occur whatever the order or time of addition of the different components to the system. This is in marked contrast to the result obtained when, in a complementary experiment, an inhibitor was produced by hydrolysis of an antibody (Porter, 1950*b*). Then it was found that a part of rabbit anti-ovalbumin would retain the power to combine with the antigen but the reaction was reversible in either direction.

The variation in response of different antisera to addition of inhibitor emphasizes the complex



Comparison of behaviour of bovine-serum albumin (BSA) and inhibitor (I) on diffusion into antiserum in agar gel by the method of Ouchterlony (1953). Concentrations 5, 0·5 and 0·05 mg./ml. with both reagents. Centre cup contains rabbit anti-bovine-serum albumin. Photograph was taken after the plate had been standing for 5 days at 2°.

mixtures of different types of antibody which must be present. It is possible to list different types of antibody from the reaction of different antisera with the inhibitor and with homologous and heterologous antigens. Thus there is (1) antibody unable to react with inhibitor or goat-serum albumin; (2) antibody which precipitates with inhibitor; (3) antibody which will combine but will not precipitate with inhibitor; (4) antibody which will combine with goat-serum albumin but not with inhibitor; (5) antibody which will combine with inhibitor but not with goat-serum albumin; (6) antibody which will combine with both inhibitor and goat-serum albumin.

By using other heterologous antigens further subdivision could be made, and it seems that all antisera contain a complex mixture of antibodies directed against different combining sites and different combinations of combining sites in the antigen. It is possible that properties of antisera such as avidity could be interpreted in terms of the proportions of antibodies directed against different numbers and types of antigenic combining sites. It is unlikely that any two antisera will be identical in the amounts of the different types which they contain, though the trend which has been observed of an increase in the proportion of antibodies able to react with a given site on continued immunization may be generally true.

This is similar to the observation often made that the cross-reaction of antisera with a heterologous antigen increases with continued immunization. Two explanations of this phenomenon have been offered. One is that the antibodies formed are directed against an increasing number of antigenic combining sites, some of which are common to the heterologous and homologous antigens. The other is that the antibody-combining sites become a less exact fit for the antigen sites, and hence combination with similar but not identical sites becomes possible. As a very similar phenomenon has now been observed with a part of the homologous antigen containing one or more of the original combining sites, the first explanation seems to be more probable. It would follow that a distinction should be made between the potential combining sites of an antigen and the actual combining sites in relation to a particular antiserum or antibody fraction.

The observation that the inhibitor is able to provoke anaphylactic shock in a passively sensitized guinea pig is in contrast with an earlier observation by Humphrey (unpublished). He found that the fragment of anti-ovalbumin which retains the power to combine with ovalbumin (Porter, 1950*b*) will not passively sensitize a guinea pig to shock by subsequent injection of ovalbumin. The mechanism of anaphylactic shock is not understood, but it is possible that attachment of the antibody to certain tissues is important and that the whole molecule is necessary for this to occur. When an antigen is injected it will presumably remain in solution until meeting the antibody, and only its power to react is being tested. The observation with the fragment of anti-ovalbumin that although it retained the power to combine with ovalbumin it could not sensitize a guinea pig to anaphylactic shock suggests that, though some biological activities may be a function of only a part of the molecule, others, *in vivo*, may necessitate the whole molecule. For example, the inhibitor, though still able to combine with antibody, may have lost its power to stimulate antibody formation when injected into a rabbit; owing to the amount of material required for the test this has not been investigated so far.

SUMMARY

1. Chymotryptic digestion of bovine-serum albumin in a dialysis sac produced a diffusible digestion product which retained the power to combine with rabbit anti-bovine-serum albumin.

2. This product has been isolated, principally by zone electrophoresis. The *N*-terminal amino acid is phenylalanine.

3. The molecular weight of the active fragment is about 12 000, but it readily dimerizes, probably through a free sulphydryl group.

4. The reaction of this substance with different rabbit antisera has been studied and the results used to classify the different types of antibody which appear to be present in all antisera.

5. This active fragment can provoke anaphylactic shock in a guinea pig passively immunized by injection of rabbit anti-bovine-serum albumin.

I wish to thank Dr J. H. Humphrey for suggesting and carrying out the anaphylactic-shock experiments, Dr P. A. Charlwood for studying the molecular weight of inhibitor, Dr S. Jacobs for the amino acid analysis of the inhibitor and Dr G. H. Beaven for estimation of the tryptophan content; I also wish to thank Miss J. Morgan for her invaluable technical assistance throughout.

REFERENCES

Archibald, W. J. (1947). *J. phys. Chem.* **51**, 204.
Beaven, G. H. & Holiday, E. R. (1952). *Advanc. Protein Chem.* **7**, 319.
Charlwood, P. A. (1957). *Trans. Faraday Soc.* **53**, 871.
Craig, L. C. & King, T. P. (1955). *J. Amer. chem. Soc.* **77**, 6620.
Craig, L. C. & King, T. P. (1956). *J. Amer. chem. Soc.* **78**, 4171.
Crewther, W. G. & Lennox, F. G. (1950). *Nature, Lond.*, **165**, 680.
Elliot, S. D. (1950). *J. exp. Med.* **92**, 201.
Flodin, P. & Kupke, D. W. (1956). *Biochim. biophys. Acta*, **21**, 368.
Holiday, E. R. (1939). *Proc. Roy. Soc.* A, **170**, 79.

Hughes, W. L. (1950). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 79.
Kimmel, J. & Smith, E. L. (1954). *J. biol. Chem.* **207**, 515.
Kleczkowski, A. (1945). *Brit. J. exp. Path.* **26**, 24.
Landsteiner, K. (1942). *J. exp. Med.* **75**, 269.
Landsteiner, K. & Chase, M. W. (1933). *Proc. Soc. exp. Biol., N.Y.*, **30**, 1413.
Lapresle, C. (1955*a*). *Ann. Inst. Pasteur*, **89**, 654.
Lapresle, C. (1955*b*). *Bull. Soc. Chim. biol., Paris*, **37**, 969.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Northrop, J. H., Kunitz, M. & Herriott, R. (1948). *Crystalline Enzymes*. New York: Columbia University Press.
Ouchterlony, Ö. (1953). *Acta path. microbiol. scand.* **32**, 231.
Porath, J. (1956). *Biochim. biophys. Acta*, **22**, 151.
Porter, R. R. (1950*a*). *Biochem. J.* **46**, 473.
Porter, R. R. (1950*b*). *Biochem. J.* **46**, 479.
Porter, R. R. (1953). In *The Proteins*, vol. 1B, p. 973. Ed. by Neurath, H. & Bailey, K. New York: Academic Press.
Porter, R. R. (1955). *Biochem. J.* **59**, 405.
Porter, R. R. (1957). In *Methods in Enzymology*, vol. 4. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press.
Stein, W. H. & Moore, S. (1949). *J. biol. Chem.* **178**, 79.
Straessle, R. (1951). *J. Amer. chem. Soc.* **73**, 504.

Polynucleotides. Partial Purification and Properties of a Polynucleotide Phosphorylase from *Micrococcus lysodeikticus*

By R. F. BEERS, JUN.*
Division of Biochemistry, Department of Biology, Massachusetts Institute of Technology, Cambridge 39, *Massachusetts, U.S.A.*

(*Received* 4 *October* 1956)

The discovery by Grunberg-Manago & Ochoa (1955) of a polynucleotide phosphorylase in extracts of *Azotobacter vinelandii* prompted us to examine extracts of *Micrococcus lysodeikticus* for this enzyme. The fact that the cells can be lysed under extremely mild conditions with egg-white lysozyme suggested that the problems of isolating the enzyme might be much simpler than those encountered in bacterial preparations requiring physical methods for disrupting the cell walls.

The phosphorylase was first detected in alcohol extracts of *M. lysodeikticus* in experiments done in collaboration with Dr P. Olmsted. These studies were a repetition of the original experiments described by Grunberg-Manago & Ochoa (1955). Aqueous extracts catalysed the formation from ^{14}C-labelled adenosine 5′-diphosphate (ADP), in the presence of magnesium and at pH 8, of a ^{14}C-labelled substance which failed to move on paper chromatography with the solvent [*iso*butyric acid–0·5N-aq. ammonia soln. (10:6, v/v)].

Further studies have been made on the isolation of the enzyme, on a more convenient and reliable enzyme assay and on the synthesis of large quantities of polyadenosine monophosphate [poly A or $(AMP)_n$]. Brief notes on various phases of the research have been published elsewhere (Beers, 1956*a*, *b*, *c*).

* Present address: Robert W. Johnson Laboratory, Bowles Research Fund, Children's Hospital School, Baltimore 11, Maryland, U.S.A.

MATERIALS

The sodium salts of ADP, cytidine 5′-diphosphate, uridine 5′-diphosphate and guanosine 5′-diphosphate were obtained from Pabst Laboratories, Milwaukee, Wisconsin. Inosine 5′-diphosphate was obtained from Sigma Chemical Co., St Louis, Missouri.

METHODS

Preparation of cells. A strain of *Micrococcus lysodeikticus*, no. 4698, obtained from the American Type Culture Collection, was grown in aerated submerged cultures according to the method of Beers (1955). The cells were harvested after 48 hr. growth by means of the Sharples centrifuge. From 20 l. of medium between 50 and 75 g. (dry wt.) of cells was usually obtained. These were washed three times with water and stored frozen in a deep-freeze.

Preparation of the enzyme. The frozen cells were thawed and mixed with 2 vol. of 0·5% NaCl in a Waring Blendor at low speed. The pH was adjusted to 8·0 with KOH, and for each 50–75 g. (dry wt.) of cells the white of one egg was added to the suspension of cells and mixed thoroughly without frothing. (Control studies have shown no phosphorylase activity in egg white or crystalline lysozyme.) Lysis was allowed to proceed at room temperature without further agitation. Lysis, which began promptly, was accompanied by a gradual thickening in the consistency of the cell mixture, a change in the colour from canary-yellow to yellow–green and the production of a mercaptal odour. Eventually, the preparation became a thixotropic gel. Lysis was considered to be complete when the preparation failed to adhere to the glass walls of the container. The entire process took 1–2 hr. Failure to observe these changes almost always indicated that the pH was too low.