agreement with those obtained by the method of Lamm (used by Polson), not only for glycine but for material of much lower diffusion constant. It seems likely, therefore, that the difference may depend on the sample of lactoglobulin and on the method of its preparation.

The molecular weight. Estimates of the molecular weight of lactoglobulin by various methods range from 33,000, by X-ray diffraction on wet crystals of the orthorhombic form (McMeekin & Warner, 1942), to about 42,000, by sedimentation velocity and diffusion (Pedersen, 1936). The more reliable determinations by X-ray diffraction on dry crystals gave values about 36,000 (McMeekin & Warner, 1942). Pedersen's (1936) measurements on sedimentation equilibrium gave a mean value about 39,000; the variation of the value obtained with the method of computation suggests that the material used by him (and by Polson, 1939) was not homogeneous. Measurements of osmotic pressure have given values of 37,800 (Gutfreund, 1945), 37,300 ± (S.D.) 300 (Johnston & Ogston, 1946) and 35,020 ± (S.D.) 140 (Bull, 1946*a*). Bull (1946*b*) obtained a value of $2 \times 17{,}100$ from measurements of surface pressure.

The present values of the sedimentation and diffusion constants, using Pedersen's (1936) value of 0·751 for the partial specific volume, give values of 35,600 at 1 g./100 ml. and 35,400 at infinite dilution.

This wide variation of values is unsatisfactory. Errors may in some cases (as that of the sedimentation constant) have arisen from technical factors in the measurements; in others, variation may have been due to the treatment of the material during preparation, as is suggested by the data of Pedersen (1936) and the findings of Bull (1946*a*). It is difficult to decide which is the true value for the molecular weight; the most reliable determinations by X-ray diffraction favour a value near to 36,000, and our measurements on material which was very nearly homogeneous support this. The possibility exists, however, that lactoglobulin is not a material whose physical constants have unique values.

SUMMARY

1. Measurements of the sedimentation and diffusion constants of lactoglobulin are described. The values, extrapolated to infinite dilution, are S_{20} (corr.) $= 2{\cdot}83 \times 10^{-13}$ and D_{20} (corr.) $= 7{\cdot}82 \times 10^{-7}$; these differ considerably from other data but combine to give a molecular weight of 35,400. Other reliable values of the molecular weight agree with this.

2. The causes of differences between measured values of the constants of lactoglobulin are discussed.

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The Estimation of Peroxidase Activity

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The standard method for the estimation of the catalytic activity of peroxidase is that initially published by Willstätter & Stoll (1918). They allowed a measured quantity of the enzyme preparation dissolved in 2 l. distilled water to react with fixed amounts of hydrogen peroxide and pyrogallol for exactly 5 min. The reaction was then stopped by addition of sulphuric acid and the yellow purpurogallin transferred to ether in a separating funnel. The concentration of the ethereal solution was estimated by comparison with standard purpurogallin solutions. The activity of the enzyme, Purpurogallin number (*P.N.*) or *Purpurogallinzahl* (*P.Z.*) of Willstätter, was defined as the weight of purpurogallin in mg. formed by 1 mg. of the enzyme preparation. Willstätter & Weber (1926) showed that the *P.Z.* values obtained varied over a wide range with changes in the quantities of the ingredients and in the volume of solution used for the test. A similar method for estimation of *P.N.* was

used by Elliott & Keilin (1934), Keilin & Mann (1937) and by Theorell (1942).

Recently, Sumner & Gjessing (1943), having mentioned that the work on peroxidase in their laboratory was 'somewhat hampered by lack of a satisfactory means of determining its activity', have described a new method in which the reaction is carried out in 20 ml. phosphate buffer. They state that the resulting values for activity were somewhat higher than those obtained by Willstätter's method. In the present work the effect of dilution and of buffer concentrations on the activity of different enzyme preparations has been investigated, and the

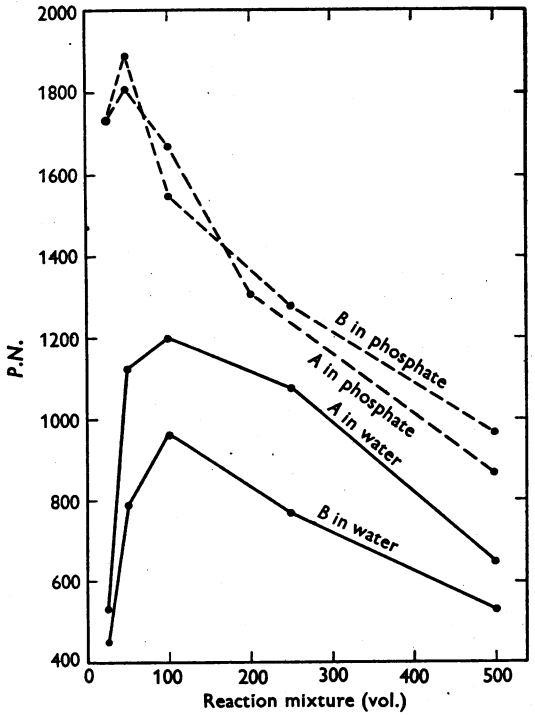

Fig. 1. Effect of varying the volume of the reaction mixture on the activity of two highly purified peroxidase preparations, A and B, in water and in 0·07 M-phosphate buffer pH 5·9. Reaction carried out in presence of 12·5 mg. H_2O_2 and 1·25 g. pyrogallol at 20°.

method devised by Willstätter and his co-workers compared with that of Sumner & Gjessing. The colorimetric methods have also been compared with a new manometric method based upon the formation of carbon dioxide during the catalyzed oxidation of pyrogallol.

EXPERIMENTAL

Methods

Enzyme preparations of widely differing purity were examined including the crude press juice of horse-radish root, several highly purified preparations isolated by the methods of Elliott & Keilin (1934) and Keilin & Mann (1937), and a number of fractions of intermediate activity obtained during the fractionation of the crude juice by the same and by other methods. The reagents used were of A.R. standard, the pyrogallol being resublimed; the use of glass-distilled water was found to be essential. All experiments were carried out at room temperature (*c.* 20°). The King photoelectric colorimeter was calibrated for purpurogallin by means of standard solutions of the twice recrystallized pigment in peroxide-free ether using an Ilford blue filter no. 302.

Willstatter's *P.Z.* estimation was modified slightly, being carried out in 500 ml. water in presence of 12·5 mg. H_2O_2 and 1·25 g. pyrogallol (Keilin & Mann, 1937). The amount of enzyme, added at zero time, was adjusted in order that not more than 10 mg. purpurogallin were formed in 5 min. The yellow ethereal extract was dried with Na_2SO_4 before being examined in the colorimeter.

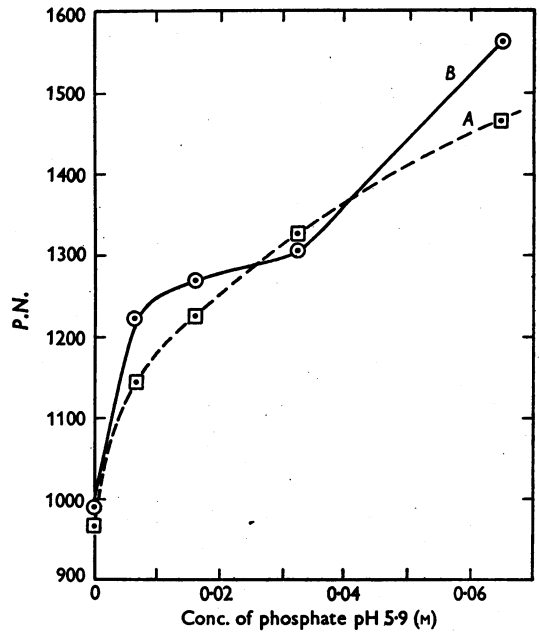

Fig. 2. Effect of different concentrations of phosphate buffer on the activity of two highly purified peroxidase preparations, A and B. Reactions carried out in a volume of 100 ml. in presence of 12·5 mg. H_2O_2 and 1·25 g. pyrogallol at 20° and pH 5·9.

Influence of volume of reaction mixture and of phosphate buffer on the results obtained by Willstätter's method

Willstätter carried out his purpurogallin test at high dilutions in order to prevent destruction of enzyme by H_2O_2, but, as Sumner & Gjessing (1943) have shown, high enzyme activity can be obtained in more concentrated solutions. Fig. 1 shows the fluctuation in the *P.N.* values of two highly purified enzyme preparations observed when the total volume of the reaction mixture was varied between 25 and 500 ml. The conditions of the test were otherwise as described above. A maximum value for enzyme activity was

obtained in 100 ml. water. When purified peroxidase preparations are used the concentration of protein in the test reaction is very low, and under these conditions denaturation may occur (Cohn & Edsall, 1943). In the presence of salts, which will increase the stability of the highly diluted protein and at the same time control the pH of the solution on which the reaction velocity is very dependent, higher and more reproducible values for *P.N.* might be expected. Addition of phosphate buffer pH 5·9 to give a final concentration of 0·07 M in the experiments just described yielded considerably higher figures for enzyme activity and the optimum volume was about 50 ml. (Fig. 1). The effect of changing the concentration of phosphate, while maintaining the pH at 5·9 and the volume at 100 ml., is shown in Fig. 2 where the *P.N.* is seen to increase with concentration of buffer. Thus, while Willstätter's conditions are far removed from the optimum, those of Sumner & Gjessing are very close. The risk of destruction of enzyme by high concentration of H_2O_2 has, therefore, been exaggerated by Willstätter: it is not until the volume of the reaction mixture falls below 100 ml. that the destruction becomes considerable while, in presence of phosphate buffer, the harmful effect of H_2O_2 is greatly reduced.

A comparison of P.N. determinations by the methods of Willstätter and of Sumner & Gjessing

The data on peroxidase activity in the literature are almost exclusively expressed in terms of Willstätter's *P.Z.* It is, therefore, important that the quantitative relationships between this and any new method should be determined in order that the values obtained by the new method may be translated to Willstätter's scale. With this object in view the comparative measurements summarized in Table 1

Table 1. *A comparison of the methods of Willstätter and of Sumner & Gjessing for the determination of enzyme activity of different peroxidase preparations*

Sample	Willstätter *P.N.*	Sumner & Gjessing *P.N.*	*P.N.* ratio
No. 1 (purified)	735	1080	0·68
No. 2 (purified)	508	1460	0·35
	Purpurogallin formed, mg./ml. peroxidase preparation		
Horse-radish press juice	68	100	0·68
Horse-radish press juice, filtered	107	203	0·53
Two fractions isolated from press juice	280	810	0·35
	83	228	0·36

were made. Sumner & Gjessing's method is as follows: 2 ml. 5% pyrogallol, 2 ml. 0·5 M-phosphate buffer pH 6·0, 15 ml. water and 1 ml. 1% H_2O_2 are mixed in a 125 ml. Erlenmeyer flask at 20°. Suitably diluted peroxidase (1 ml.) is added, and after 5 min. the reaction is stopped by addition of 1 ml. 2 N-H_2SO_4. The purpurogallin is extracted with ether and estimated in a colorimeter. All the determinations in Table 1 were made in duplicate, when agreement within experimental error was obtained. The recurrence of two values of the *P.N.* ratio (last column) is striking but inexplicable, since each ratio (0·68, 0·35) was observed in the cases of both pure and crude preparations.

Manometric methods for estimation of peroxidase activity

The course of the oxidation of pyrogallol to purpurogallin was studied by Willstätter & Heiss (1923). According to them the overall reaction is

$$2C_6H_6O_3 + 3H_2O_2 = C_{11}H_8O_5 + 5H_2O + CO_2,$$

hence the formation of 1 mg. purpurogallin should give rise to 102 μl. CO_2. Experiments were carried out at 20° in Barcroft differential manometers with flasks which were fitted with side bulbs for the delivery of acid. The concentrations of the ingredients in the right-hand flasks of the manometers were similar to those used in the method of Sumner & Gjessing for *P.N.* determination, but the volume was reduced from 20 ml. to 2·3 ml. Thus the right-hand flasks received 0·2 ml. 5% pyrogallol, 0·5 ml. 0·25 M-phosphate buffer pH 5·9 and 1·5 ml. water. In a dangling tube, suspended from the central tube of the flask, was placed 0·1 ml. peroxidase preparation while the side bulb received 0·5 ml. 20% H_2SO_4. The left-hand flasks received all the reagents except peroxidase.

In order to rule out the possibility of O_2 uptakes by crude enzyme preparations the flasks were evacuated and refilled with N_2 containing 5% CO_2. With purified preparations the same results were obtained in this gas mixture as in air. The flasks were equilibrated in the manometer bath for 10 min. and after closing the taps the peroxidase was added. At 5 min. the CO_2 output was recorded and the acid immediately added from the side bulbs. The purpurogallin formation was calculated from the CO_2 evolved, and was checked by colorimetric examination of the ethereal extracts of the solutions in the flasks. As is seen in Table 2A there is good agreement between the results. In further experiments the *P.N.* of a preparation was estimated in two samples by the manometric method and also by the method of Sumner & Gjessing. Both crude and pure peroxidase fractions yielded results in good agreement (Table 2B

Table 2. *Comparison of manometric and colorimetric methods for estimation of peroxidase*

(Preparation no. 1 (0·0016 mg.) and press juice (1·88 mg. dry wt.) were used in these estimations.)

Enzyme	A. Estimations of purpurogallin formed from pyrogallol by peroxidase: Manometrically by CO_2 production (mg.)	Colorimetrically (mg.)
Preparation no. 1	1·97	2·09
Filtered press juice of horse-radish root	2·15	2·25
	B. Estimations of peroxidase activity by two methods: Manometrically by CO_2 production (*P.N.*)	Colorimetrically (*P.N.*)
Preparation no. 1	1250	1370
Filtered press juice of horse-radish root	1·34	1·28

Further experiments on CO_2 production during the formation of pyrogallol were carried out in a Brinkman apparatus as used by Meldrum & Roughton (1934) for the estimation of carbonic anhydrase. In this method the reaction mixture is shaken very rapidly in order to maintain equilibrium between the gas phase and the dissolved gases during rapid reactions. A modified apparatus was used for this work in which the manometer was replaced by a manometric capsule incorporating a very thin diaphragm. Movements of the latter were recorded by means of an optical lever and mirror. With this arrangement there is virtually no time lag between the formation of a gas and its recording by the optical lever. Reaction mixtures of the type used in the Barcroft manometers were tested in this apparatus, and, with rapid shaking, a close proportionality between quantity of enzyme taken and rate of CO_2 evolution was observed over a period of 40 sec. After this time denaturation of the enzyme is likely to occur.

The experiments just described show that manometric determinations of peroxidase activity are possible.* The close agreement between this method and the colorimetric method as modified by Sumner & Gjessing enables peroxidase activity to be expressed in terms of Q_{CO_2} and hence allows *P.N.* values to be converted to the commonly used Q notation. Thus $P.N. = 1$ corresponds to $Q_{CO_2} = 102 \times 12 = 1224$.

Observations on the stability of peroxidase preparations

Experience gained during the present investigation confirms the observations of Willstätter and of other workers on the large and unaccountable variations of about 30% which are obtained when the *P.N.* of a peroxidase preparation is tested periodically. A possible explanation of these variations was the lack of buffering capacity in the Willstätter method, but an investigation, in which the *P.N.* of a very active peroxidase was tested at intervals during 7 weeks in presence of phosphate by the method of Sumner & Gjessing, did not confirm this view. In spite of the control of pH the *P.N.* varied haphazardly between 1160 and 1780 although duplicate simultaneous determinations were in good agreement. These experiments confirm the view that such variations are due not to any changes in the peroxidase preparation, which would give rise to a progressive change in *P.N.*, but rather to minute traces of impurities, possibly heavy metals, in the reagents.

* The manometric method described above is fundamentally different from that devised by Altschul & Karon (1947) which consists of the estimation of unused H_2O_2 in terms of O_2 liberated by catalase.

SUMMARY

1. The methods for the determination of peroxidase activity based upon the catalyzed oxidation of pyrogallol to purpurogallin by hydrogen peroxide have been re-examined.

2. The effects of varying concentrations of reactants and of phosphate buffer have been investigated with a view to the development of a method giving more reproducible results.

3. A manometric method, based upon the evolution of carbon dioxide during the oxidation, has been devised which gives results in good agreement with more recent methods involving colorimetric determinations of purpurogallin. It thus becomes possible to express the activity of peroxidase preparations in terms of Q_{CO_2}.

4. The periodic fluctuations in apparent peroxidase activity of enzyme preparations, the origin of which still remains obscure, have been confirmed.

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