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The Oxidation of Myoglobin to Metmyoglobin by Oxygen. 1

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The study of the oxidation of haemoglobin and myoglobin to methaemoglobin and metmyoglobin by molecular oxygen is important in understanding the stability of oxyhaemoglobin and oxymyoglobin.

Brooks (1931, 1935) investigated the oxidation of haemoglobin (Hb) to methaemoglobin and found that under constant conditions of oxygen pressure, pH, salt concentration and temperature the reaction was first order with respect to the concentration of unoxidized haemoglobin, and that the rate of oxidation had a well defined maximum value at a low oxygen pressure (about 20 mm. at 30°).

Legge (1942) suggested the spontaneous decomposition of one of the oxygenation intermediates $Hb_4(O_2)_2$ as a reaction mechanism to account for these results. Later Lemberg & Legge (1949) amplified this mechanism by assuming the participation of two oxidizable XH_2 groups on the protein to make possible an intramolecular reaction fitting the stoicheiometric relation:

$$Hb_4(O_2)_2 + 2XH_2 \rightarrow Hb_4(OH)_4 + 2X. \qquad (A)$$

George (1949) put forward an alternative representation of the kinetics and discussed a possible intermolecular free radical mechanism which avoids some difficulties inherent in an intramolecular mechanism (George, 1952).

An investigation of the oxidation of myoglobin should help in deciding between mechanisms of this kind, for, since myoglobin has only one haem group per molecule, a reaction of type *A* occurring in this system should necessarily be second order. This paper deals with the general chemistry of the reaction—choleglobin and denatured protein formation—the overall stoicheiometry and the results of a kinetic study to establish this point.

In this paper the symbols Mb, MbO_2, MetMb are used to represent reduced myoglobin, oxymyoglobin and metmyoglobin respectively. Similarly, in the case of haemoglobin, the corresponding symbols Hb, HbO_2 and MetHb are used.

MATERIALS AND METHODS

Preparation of pure, recrystallized equine metmyoglobin. The method employed is based on that of Theorell (1932) and Lawrie (1951). Four hearts were usually processed. After machine-mincing, the muscle tissue was extracted with water, the solution neutralized and treated with 1/5 of its volume of saturated, basic lead acetate to remove muscle globulins and other impurities. After centrifugation, the solution was again neutralized and sufficient solid Na_2PO_4 to remove excess lead was added. The pH was once more returned to 7·0 by addition of solid NaH_2PO_4 and the solution was centrifuged. It was found particularly important to adjust the pH to 7·0 at the points indicated above in order to obtain the maximum yield. It was also desirable to keep the temperature as near 0° as possible throughout the preparation. The MetMb solution was further purified by crystallization, induced by saturation of the solution with ammonium sulphate, followed by solution in 3 M-phosphate buffer of pH 6·8. This treatment separates the MetMb from any traces of haemoglobin which may be present since haemoglobin is almost completely insoluble in phosphate buffer of this molarity (Morgan, 1935).

Lastly, the material was dissolved in the desired buffer solution (see below), traces of $(NH_4)_2SO_4$ were removed by

dialysis and the material stored at 0°. The yield averaged 1·1 g. MetMb/kg. of heart. The concentration and purity of the sample were determined by the method described by De Duve (1949).

Myoglobin solutions. The myoglobin solutions were buffered heavily as described by Brooks (1935) with K_2HPO_4 and KH_2PO_4, the total concentration of phosphate being 0·6 M and the $HPO_4^=/H_2PO_4^-$ ratio 0·159/0·841. This gives the pH as 5·69 at 30°. The use of the heavily buffered solution has the advantages of preventing both microbial contamination and change of pH during the experiment. The latter is particularly important because the oxidation rate is dependent on the H ion concentration.

Before each experiment MetMb was converted into MbO_2 by first reducing to the ferrous form by addition of a small quantity of sodium hydrosulphite. Excess hydrosulphite is rapidly oxidized by atmospheric oxygen when the flask is gently shaken, and MbO_2 is formed.

Methods of analysis. Spectrophotometric methods of analysis were employed for the determination of MbO_2 and MetMb. The instrument used was a Unicam quartz spectrophotometer. Using a wavelength of 575 mμ. the millimolar extinction coefficient (E_{mM}) was very near to 11·0 for MbO_2 and 3·0 for MetMb at pH 5·69. These values varied slightly for different preparations. The solutions obeyed Beer's Law. At the beginning of each experiment the optical density of the pure MetMb solution was recorded. The MetMb was then converted into MbO_2 and the optical density recorded.

The fraction of MbO_2 in a given solution containing a mixture of MbO_2 and MetMb was determined by comparing the optical density of the solution with the corresponding optical densities of the two solutions containing 100% MbO_2 and 100% MetMb. It was found most convenient to work with optical densities between 0·6 and 0·8 for MbO_2 with the corresponding densities of 0·16 and 0·22 for MetMb. When using solutions of higher concentrations than would give values of this order of magnitude, appropriate dilution was made before reading in the spectrophotometer.

Apparatus and experimental method. MetMb solution (15–20 ml.) at the desired dilution was placed in the reaction flask, which was boat-shaped like those used by Meldrum & Roughton (1934), but without the partition along the bottom. The flask was placed in an ice bath and a minute quantity of sodium hydrosulphite added. The solution was gently shaken until the Mb was converted into MbO_2. All oxidation experiments were carried out in air at 30° in 0·6 M-phosphate buffer, pH 5·69, using the following procedure:

The solution (5 ml.) was withdrawn from the flask by means of a pipette, the optical density measured, and the solution returned to the flask. The flask was then transferred to the thermostat at 30°. The starting point of the reaction was taken at the time when the flask was placed in the thermostat. The flask was shaken mechanically 40 times/min., producing a small wave moving backwards and forwards across the surface of the solution, thus ensuring efficient stirring and renewal of the surface. At intervals 5 ml. samples of the solution were removed from the reaction flask, the optical density measured and the solution quickly returned to the flask. When using more concentrated solutions a smaller sample was removed and the appropriate dilution made before measuring the optical density. Results were recorded as percentage MbO_2 in the mixture from the time of the start of the experiment.

Warburg manometers were used in measuring the consumption of O_2 during the autoxidation in air; $1·3 \times 10^{-3}$ M solutions of myoglobin were used and the absorption of O_2 measured after the flask had been equilibrated. Calculations showed that no significant decrease in the O_2 pressure occurred as a result of the observed O_2 absorption. The same technique was used to measure the evolution of O_2 when MbO_2 was acidified with 1·0 M-metaphosphoric acid and HCl.

Cholemyoglobin formation. Under the conditions of the experiments there was the possibility that cholemyoglobin might be formed. In order to test for this the following method was employed: two samples of myoglobin solution were taken, one immediately before an oxidation experiment was begun and the other 3 hr. later. Each sample was converted into the CO-haemochromogen by the method described by Lemberg, Legge & Lockwood (1941) and the spectrum examined over the range 500–650 mμ.

Denaturation check. In order to determine whether any denaturation of the protein occurred during the reaction, use was made of the reaction between MetMb and NaN_3. On the addition of NaN_3 to a MetMb solution it is converted into the azide-metmyoglobin compound, the spectrum of which has two strong but diffuse bands at 575 and 542 mμ. Since only the ferric derivative of the native protein forms a complex with azide (Keilin, 1936) any change in the spectrum of the azide compound would be indicative of some change in the protein. Metmyoglobin solution (5 ml.) at pH 5·69 was treated with excess NaN_3 and the spectrum examined over the range 500–650 mμ. A separate 15 ml. sample of the same solution was converted to MbO_2 and allowed to oxidize as above for 3 hr. At the end of this time the material was oxidized fully by the addition of a small quantity of potassium ferricyanide. Azide was then added and the spectrum examined as before.

RESULTS

Choleglobin and denaturation check

In 3 hr. 60% of metmyoglobin was formed from oxymyoglobin. Using the methods described above it was shown (Figs. 1 and 2) that no detectable amount of either choleglobin or denatured protein is formed.

Oxidation kinetics

The metmyoglobin formation was measured at 10 and 20 min. intervals for four initial concentrations of oxymyoglobin: 0·296, 1·18, 2·11 and $2·96 \times 10^{-4}$ M. Fig. 3 shows that the percentage oxidation in a given time is independent of the absolute concentration of myoglobin, showing that the oxidation is first order with respect to unoxidized myoglobin. A typical first order plot for the experiment when the initial concentration of myoglobin was $1·18 \times 10^{-4}$ M is given in Fig. 4, and Table 1 shows the first order constant obtained in the other experiments.

The mean value of the first order constant is $0·325 \pm 0·015$ hr.$^{-1}$.

The rates of oxidation were reproducible using the same stock solution of myoglobin, but a certain amount of variation was observed between different

preparations of myoglobin and when the sample had been recrystallized. Table 2 gives values of k for seven different samples of myoglobin obtained in the course of 18 months' work. These values show a variation of some 7 % which is to be compared with an uncertainty of about 5 % in the value of an individual constant obtained from the first order plot.

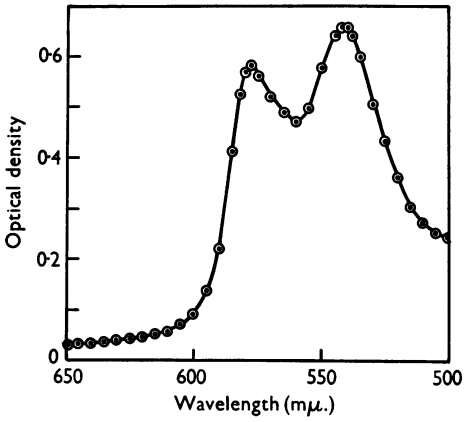

Fig. 1. Spectra of the carbon monoxide-protohaemochromogen complex formed before and after autoxidation in air at 30° in 0·6 M-phosphate buffer of pH 5·69. —, from $5{\cdot}1 \times 10^{-5}$ M-MetMb; ⊙, from a solution of the same concentration after autoxidation for 3 hr.

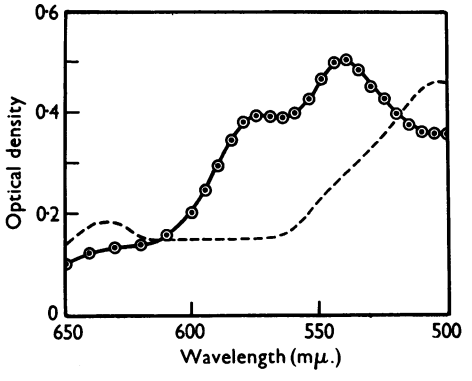

Fig. 2. Spectra of the azide-metmyoglobin complex formed before and after autoxidation of myoglobin in air at 30° in 0·6 M-phosphate buffer of pH 5·69. —, from $5{\cdot}1 \times 10^{-5}$ M-MetMb; ⊙, from a solution of the same concentration after autoxidation for 3 hr.; – – –, spectrum of acid metmyoglobin at the same concentration.

The variation between the several samples is not so marked as that found by Brooks (1931), working on the oxidation of haemoglobin, using laked blood, when in air at 25°, 0·2 M-phosphate buffer solution of pH 6·29, $k = 1{\cdot}54 \pm 0{\cdot}28 \times 10^{-2}$ hr.$^{-1}$, i.e. an 18 % variation. This comparison suggests that the variation may be due, in part, to the presence of other oxidizible substances present to a lesser

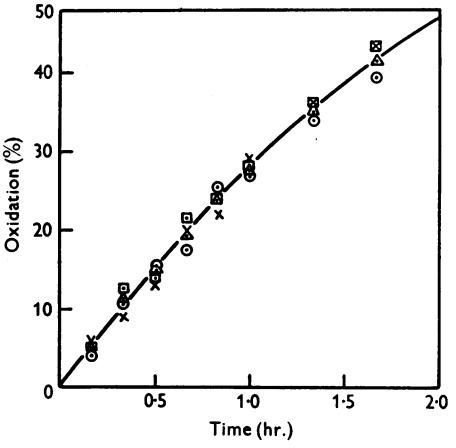

Fig. 3. The percentage formation of metmyoglobin during the autoxidation in air at 30° in 0·6 M-phosphate buffer of pH 5·69 of four solutions of different initial concentrations of MbO_2. The points ⊙, △, ⊡ and × refer to solutions of 0·296, 1·18, 2·14 and $2{\cdot}96 \times 10^{-4}$ M-myoglobin respectively.

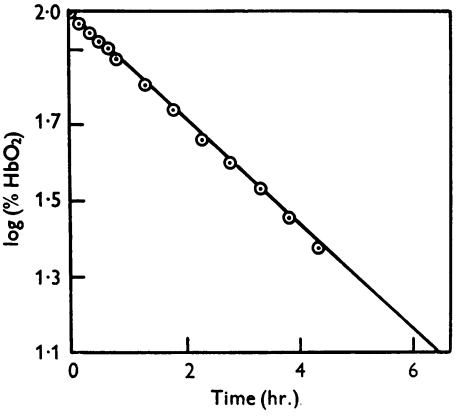

Fig. 4. A first order plot for metmyoglobin formation from spectrophotometric measurements. Initial concentration of MbO_2, $1{\cdot}18 \times 10^{-4}$ M.

Table 1. *First order rate constant for the oxidation of myoglobin to metmyoglobin at four different initial concentrations of unoxidized myoglobin, in air at* 30° *in* 0·6 M-*phosphate buffer*, pH 5·69

(Spectrophotometric methods of analysis.)

Initial concentration of unoxidized myoglobin (mol. $\times 10^{-4}$/l.)	First order rate constant k (hr.$^{-1}$)
2·96	0·34
2·11	0·34
1·18	0·32
0·296	0·30
Mean	**0·325** ± 0·015

degree in the purified myoglobin preparations used in the present experiments. This will be discussed later in relation to the results obtained in the next section.

Table 2. *First order rate constant for the oxidation of myoglobin to metmyoglobin in air at* 30° *in* 0·6 M-*phosphate buffer*, pH 5·69, *using several samples of myoglobin*

(Spectrophotometric methods of analysis.)

No. of sample	First order rate constant k ($hr.^{-1}$)
2*	0·32
3	0·38
4	0·26
4†	0·24
5	0·24
5†	0·28
6	0·25
Mean	0·27 ± 0·02

* Mean of four determinations.
† Recrystallized sample.

Stoicheiometry of the autoxidation

Measurements of the oxygen absorption of small quantities of $1·3 \times 10^{-3}$ M-myoglobin, in air at 30° in 0·6 M-phosphate buffer, pH 5·69, using Warburg manometers showed that about 1·5 moles oxygen are absorbed for each mole metmyoglobin formed. Typical results are shown in Table 3. The final volume of oxygen absorbed was estimated by extrapolation, with a probable error of about 5 %. The reliability of this extrapolated value is supported by plotting the absorption data as a first order reaction, taking this value as the end point. A linear plot is obtained as shown in Fig. 5 for the data relating to Exp. 2 in Table 3; the first order constant of 0·29 $hr.^{-1}$ is in good agreement with the value obtained by spectrophotometric measurements of the metmyoglobin formed. The reproducibility of these oxygen absorption experiments was not particularly good. Exp. 2, using 2 ml. of solution, gave oxygen uptakes of 106, 70 and 90 μl. when repeated, and the uncertainty in the ratio moles oxygen absorbed/moles metmyoglobin formed is thus 0·3. These absorption values were unaffected if the centre cup of the Warburg flask did not contain sodium hydroxide. It therefore appears that no carbon dioxide is evolved in the reaction. Even though the total quantity of oxygen absorbed varies between these rather wide limits the first order constant obtained by the method given above (Fig. 5) was still identical with that found in the spectrophotometric measurements. No significant difference was observed in either the total quantity of oxygen absorbed or the first order constant when the flask was filled with oxygen or with air.

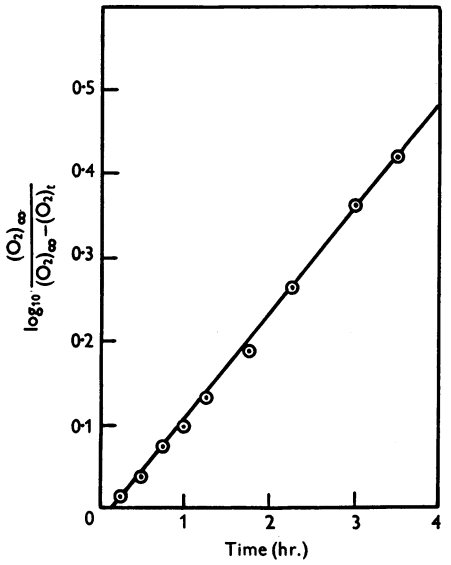

Fig. 5. First order plot for metmyoglobin formation from oxygen absorption data. Initial concentration of MbO_2, $1·3 \times 10^{-3}$ M.

For myoglobin the half saturation pressure of oxygen is of the order 1–2 mm. and so in air about 99 % is present as oxymyoglobin. If the stoicheiometry corresponded to the hypothetical figure for the oxidation of the ferrous compound

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$$

three-quarters of the bound oxygen in oxymyoglobin should be evolved on autoxidation. The measured absorption of 1·5 ± 0·3 moles oxygen for each mole of metmyoglobin formed means that this quantity of oxygen is used up in the reaction in addition to all the bound oxygen originally present as oxymyoglobin and the stoicheiometry for the autoxidation is thus

1 mole Mb + 2·5 ± 0·3 moles O_2 → 1 mole MetMb.

Table 3. *The oxidation of* $1·3 \times 10^{-3}$ M-*myoglobin to metmyoglobin in Warburg flasks in air at* 30° *in* 0·6 M-*phosphate buffer of* pH 5·69

Exp.	Volume of solution (ml.)	Oxygen absorbed (extrapolated value) (μl.)	Mol. O_2 absorbed / Mol. MetMb formed
1	4·5	210 ± 15	1·60 ± 0·11
2	2·0	90 ± 5	1·54 ± 0·08

The overall chemical equation for the autoxidation will be given by this expression or some multiple of it.

Comparable data are not available for haemoglobin, but Roaf & Smart (1923) have shown that when oxyhaemoglobin is denatured by acidification about 50% of the bound oxygen is liberated. It appears that the remainder is used in oxidizing the haem to haemin and oxidizing hydrogen donor groups on the protein part of the molecule (Lemberg & Legge, 1949).

Acidification of oxymyoglobin, in the Warburg apparatus, with 1·0M-metaphosphoric acid gave no measurable evolution of oxygen, and with 1·0N-hydrochloric acid only 11% of this bound oxygen was released.

DISCUSSION

The oxidation of myoglobin to metmyoglobin by oxygen at pH 5·69 in the absence of acceptors is a relatively simple reaction. In contrast to the experiments of Lemberg *et al.* (1941), on the reaction of oxyhaemoglobin with ascorbic acid, no detectable amount of choleglobin is formed, and unlike the reaction which occurs when acid or pyridine is present, where denaturation accompanies oxidation, the protein remains in a native condition.

The results of the stoicheiometric experiments do, however, suggest that the protein is not in its original state, for $2·5 \pm 0·3$ moles oxygen are used up for each mole of metmyoglobin formed. The simplest stoicheiometry for the autoxidation of a ferrous compound involves four equivalents of ferrous compound for each oxygen molecule; thus additional reactions absorbing oxygen accompany the oxidation of the haem in myoglobin. The similarity between the kinetics of oxygen absorption and metmyoglobin formation argues against this oxygen being used to oxidize traces of impurity, and suggests that hydrogen donor groups on the protein molecule itself are involved. In this respect the experiments of Mirsky & Anson (1936) are particularly interesting. They showed that tyrosine and tryptophan groups in proteins were oxidized by even mild reagents like potassium ferricyanide, and that with tyrosine itself one mole could react with at least 2·6 moles ferricyanide. Myoglobin contains two molecules each of tyrosine and tryptophan per molecule (Tristram, 1949). There are thus, in the myoglobin molecule, four known groups susceptible to oxidation, and if the mechanism for the oxidation of the haem involves intermediates which are strong oxidizing agents it is possible that other amino-acid residues could also be attacked.

The participation of hydrogen-donor groups on the protein in the autoxidation mechanism has been suggested by Lemberg & Legge (1949), but for a different reason. Legge (1942) showed that over a certain range of oxygen pressures Brooks's data on haemoglobin autoxidation (1931, 1935) could be explained if the reaction involved the spontaneous decomposition of the oxygenation intermediate $Hb_4(O_2)_2$ in an intramolecular reaction. To account for this, Lemberg & Legge postulated two oxidizable XH_2 groups in the haemoglobin molecule to complete the stoicheiometry in an equation

$$Hb_4(O_2)_2 + 2XH_2 \rightarrow Hb_4(OH)_4 + 2X,$$

thus accounting for the full oxidizing capacity of the two oxygen molecules. The probable participation of hydrogen-donor groups on the protein, suggested by our experiments on oxygen absorption during the autoxidation of myoglobin, leads to a conclusion opposite to that of Lemberg & Legge. Since myoglobin contains only one haem group per molecule the consumption of 2·5 moles of oxygen for each mole of metmyoglobin formed must mean that the reaction is intermolecular.

It is clear that the production of metmyoglobin under the above conditions is not a straightforward autoxidation of a ferrous compound, but is a coupled oxidation. However, it differs in three important respects from the coupled oxidations studied by Lemberg *et al.* (1941). First, it does not appear to involve an added hydrogen donor like the ascorbic acid in Lemberg's experiments. Secondly, the metmyoglobin is not reduced by the hydrogen donor as methaemoglobin is reduced by ascorbic acid, and, as a consequence, there is no catalytic cycle for the continued oxidation of the hydrogen donor. Thirdly, whereas the coupled oxidation of ascorbic acid by oxyhaemoglobin is initiated by a direct reaction between these two molecules (Lemberg *et al.* 1941), the kinetics of myoglobin autoxidation, shown above to be first order in unoxidized myoglobin, do not support a reaction of the kind:

$$MbO_2 + \text{protein} \rightarrow \text{MetMb} + \text{oxidized protein},$$

for, although this reaction could lead to a first order disappearance of myoglobin, the observed first order constant would not be independent of the absolute concentration.

By analogy with the mechanism put forward for haemoglobin autoxidation, Lemberg & Legge suggested that, in the case of myoglobin, the mechanism may be

$$Fe^{2+}.O_2.(H_2X) + Fe^{2+} \rightarrow Fe^{3+}.(OH).X + Fe^{3+}.OH,$$

and as a consequence the reaction would be second order in unoxidized myoglobin. The first order kinetics established above show that a mechanism of this type does not occur. The similarity that is now revealed between the kinetics of the autoxidation of myoglobin and haemoglobin casts doubt on the intramolecular mechanism in the case of haemoglobin, for it seems reasonable to expect that molecules so

alike in their general chemistry should react in the same way.

George (1949, 1952) discussed a free radical mechanism for haemoglobin autoxidation based on the stoicheiometric relations $4Mb \equiv O_2$ or $2Hb \equiv O_2$ which would account for the observed kinetics in the case of haemoglobin. A general mechanism of this kind needs revising, since it has now been shown that oxidation of hydrogen-donor groups is likely to be involved.

SUMMARY

1. The autoxidation of myoglobin to metmyoglobin in air at 30° in 0·6M-phosphate buffer of pH 5·69 was studied at several different concentrations and using five preparations of recrystallized myoglobin.

2. The reaction was shown to be first order with respect to unoxidized myoglobin and the mean value of the first order rate constant was $0·325 \pm 0·015$ hr.$^{-1}$.

3. Both spectrophotometric and oxygen absorption methods of analysis were used. The first order rate constants obtained by both these methods were in good agreement.

4. Under the conditions of the experiments it was shown that no detectable denaturation of the protein or choleglobin formation occurred.

5. Measurement of the oxygen absorption of the reaction showed that 2·5 moles of oxygen were used for each mole of metmyoglobin formed, strongly suggesting that hydrogen donor groups on the protein molecule are involved.

6. These results are discussed, and it is concluded that in contrast to the intramolecular mechanism of the reaction postulated by Lemberg & Legge (1949) the reaction must be intermolecular.

7. In view of the similarity of the kinetics of the autoxidation of myoglobin and haemoglobin, doubt is cast on the idea of an intramolecular mechanism in the case of the autoxidation of haemoglobin.

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The Microbiological Assay of 'Strepogenin' with *Lactobacillus casei*

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The presence of a factor in partially hydrolysed proteins, called 'strepogenin' (Sprince & Woolley, 1944), which had a growth stimulating effect for certain bacteria, has been reported by a number of investigators (Woolley, 1941; Sprince & Woolley, 1945; Pollack & Lindner, 1943; Smith, 1943; Wright & Skeggs, 1944; Wright, Fruton, Valentik & Skeggs, 1950). Woolley (1946) suggested that the peptide-like growth factor was a derivative of glutamic acid.

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A serylglutamic acid peptide from enzymic digests of casein stimulated *Lactobacillus casei* (Verdier & Ågren, 1948; Ågren, 1949). However, a number of synthetic peptides containing glutamic acid were either inactive or had a negligible activity (Woolley, 1948; Krehl & Fruton, 1948).

Dunn & McClure (1950) suggested recently that the strepogenin activity may be due to an unspecific effect of peptides, since the organism may use an essential amino-acid more readily in the bound than in the free form. Peptides of amino-acids were shown to be used by bacteria (Riesen *et al.* 1947;