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 stoichiometric 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 $0.325 \pm 0.015 \text{ hr.}^{-1}$.

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;

Simmonds, Tatum & Fruton, 1947; Krehl & Fruton, 1948), but no stimulatory response was obtained with synthetic di- and tri-peptides containing leucine, valine, glycine or alanine (Ågren, 1947, 1948).

Glutamine and glutathione have been reported to have strepogenin-like activity (Woolley, 1948). The former could be differentiated from strepogenin by its heat lability (Pollack & Lindner, 1943; Woolley, 1946, 1948). Furthermore, Wright & Skeggs (1944) pointed out that the activity of enzymic digests of casein could not be accounted for on the basis of their glutamine content.

The present experiments were undertaken to investigate the specificity of the strepogenin effect and its distribution in biological materials. A satisfactory assay technique was developed using a modified medium described for the assay of B-vitamins (Clegg, Kodicek & Mistry, 1952). For preliminary communication, see Mistry & Kodicek (1951).

MATERIALS AND METHODS

Strepogenin standard (IA37°). A partial hydrolysate of insulin instead of the Wilson liver fraction L (Sprince & Woolley, 1944, 1945) was used as the strepogenin standard since it was shown to be a rich source of this growth stimulatory factor. In preliminary trials the treatment described below was found to liberate the maximum activity. Digestion for a longer period or at lower temperature resulted in decreased activity.

Table 1. *Basal medium for the assay of strepogenin activity*

Constituent	Amounts/10 ml. single strength medium
Casein (Labco), H ₂ SO ₄ -hydrolysed*	50 mg.
Glucose	300 mg.
Potassium acetate	300 mg.
DL-Alanine	1 mg.
L-Asparagine	1 mg.
L-Cystine	2 mg.
DL-Tryptophan	2 mg.
Adenine, guanine, uracil and xanthine: each	0.1 mg.
Aneurin chloride hydrochloride	10 µg.
Riboflavin	10 µg.
Nicotinic acid	10 µg.
Pyridoxin hydrochloride	25 µg.
Calcium-D-pantothenate	10 µg.
p-Aminobenzoic acid	2 µg.
Biotin (free acid)	0.04 µg.
Pteroylglutamic acid	0.1 µg.
K ₂ HPO ₄ and KH ₂ PO ₄ : each	25 mg.
Salt solution E*	0.05 ml.

* Clegg *et al.* (1952).

200 mg. crystalline bovine insulin (Boots Pure Drug Co. Ltd.) were hydrolysed for 3 hr. at 37° in 5 ml. of conc. HCl. The acid was carefully removed under reduced pressure on a water bath and the solids dissolved in about 45 ml. of water. The pH was adjusted to 6.8–7.0 with 40% KOH, the volume made up to 50 ml., and the solution filtered and stored at 4° under toluene. Its concentration will be referred to in terms

of the original weight of insulin. This preparation was assigned a potency of 100.

Basal medium. The medium previously described (Clegg *et al.* 1952) was modified by omitting Norite-treated peptone and adding L-asparagine (Table 1).

Bacteriological procedure. *Lb. casei*. A.T.C.C. No. 7469, was used throughout this study as the test organism. The maintenance of the culture and other bacteriological techniques have been described previously (Clegg *et al.* 1952).

The inoculum was standardized to contain 30 µg. of dry wt. of bacteria per ml. and one drop was seeded aseptically into 10 ml. medium. The seeding contained 1 µg. of dry wt. of bacteria, i.e. about 1×10^6 viable cells as determined by the plate count method.

The assay tubes were incubated at 37° for 18 hr. and the resulting growth was measured turbidimetrically against the corresponding blank in a single-cell photoelectric colorimeter, using a neutral grey filter. The results were calculated from a calibration curve for *Lb. casei* in terms of dry wt. bacteria.

Choice of the size of inoculum. It was pointed out by Sprince & Woolley (1944, 1945) that the use of a small inoculum was important in the assay of strepogenin, since with larger seedings the responses due to strepogenin additions were less marked. Similar results were obtained in the present study. The medium (10 ml.) was seeded with inocula of varying strength, increasing in geometric progression. Within a range of 0.275–1.1 µg. of inoculum, the ratio of the growth stimulation by strepogenin to the concentration of inoculum, remained constant. At higher concentrations, the blanks increased relatively more than the responses obtained with the strepogenin preparation. Therefore, an inoculum containing 1 µg. of dry wt. of bacteria was adopted.

Fig. 1. Effects of various substances on the rate of growth of *Lb. casei* (18 hr. incubation) compared with the strepogenin standard, IA37°.

RESULTS

Effect of various substances on the growth of Lb. casei
A number of substances have been reported to stimulate the growth of *Lb. casei* and they were therefore reinvestigated with the use of the medium from which asparagine was omitted.

It will be seen from Fig. 1 that glutathione and glutamic acid had no stimulatory effect. Glutamine, added after autoclaving the medium, gave a small

Fig. 2. Dose response curves of the streptogenin standard, IA 37°, ●; insulin, Novo, free of hyperglycaemic-glycogenolytic factor, ○; trypsin, Armour, ▲; and plasma albumin, bovine, Armour, ×.

Fig. 3. Dose response curves of the streptogenin standard, IA 37°, ●; insulin, sheep, Boots Pure Drug Co. Ltd., ▲; chymotrypsin, Armour, ○; tobacco mosaic virus, ×.

Table 2. *Streptogenin activity of insulin, liver and yeast preparations*

Material*	Relative streptogenin activity (%)
Insulin, bovine;† streptogenin standard, IA 37°	100
Insulin, bovine;† unhydrolysed	11
Insulin, bovine;† different batch	112
Insulin, bovine;† recrystallized	95
Insulin, bovine;† hydrolysed with chymotrypsin	103
Insulin, pig†	412
Insulin, sheep†	400
Insulin, Novo, free of hyperglycaemic-glycogenolytic factor	90
Hepamino, proteolysed liver powder, Evans; unhydrolysed and hydrolysed	20†
Liver fraction L, Wilson; unhydrolysed and hydrolysed	2†
Marmite, yeast extract; unhydrolysed	6

* Except where mentioned, all materials were hydrolysed with conc. HCl for 3 hr. at 37° and tested at three or more levels.

† These preparations were obtained from Boots Pure Drug Co. Ltd.

‡ Values denote activity not strictly comparable to the streptogenin standard. Activities were calculated at 3.5 and 50 mg. per 10 ml. medium for Hepamino and liver fraction L respectively.

growth response. However, the response was considerably below that obtained with the streptogenin standard. Asparagine and aspartic acid also had a small stimulatory effect during the 18 hr. test period. In the medium used for assay, i.e. with asparagine

present, no further stimulation of growth was observed with either glutamine or aspartic acid. Kodicek & Mistry (1949) noted that the presence of asparagine greatly enhanced the effect of streptogenin preparations and therefore it was included in the medium. A detailed study of these effects will be reported in another communication.

Table 3. *Streptogenin activity of purified proteins*

Material*	Relative streptogenin activity (%)
Insulin, bovine, Boots Pure Drug Co. Ltd.; streptogenin standard, IA 37°	100
Pepsin, Armour, 1:10000 potency; unhydrolysed	19
Pepsin, Armour, recrystallized, 1:60000; unhydrolysed	6
Pepsin, Armour, 1:10000	39
Pepsin, Armour, recrystallized, 1:60000	160†
Peptides 16P obtained on recrystallization of pepsin, Armour, 1:10000	17
Trypsin, Armour	48
Chymotrypsin, Armour	153†
Turnip yellow mosaic virus	86†
Tobacco mosaic virus	56†
Myosin, rabbit	104†
Lactoglobulin, crystalline	49†
Plasma albumin, bovine, Armour	45
Egg albumin	19
Crude protein from degraded wool	13
Globin, horse	10
Globin, human	9
Globin, cow	5
Haemoglobin, bovine, Armour	5
Pea globulin	4
Edestin	2
Gelatin	1
Salmin sulphate, British Drug Houses Ltd.	0
Casein, vitamin-free, Labco	5
Casein, vitamin-free A/E, Glaxo	2
Casein, vitamin-free LW, Glaxo	2
Casein, vitamin-free, Hopkin & Williams	2
Casein, hydrolysate, vitamin-free, Ashe; unhydrolysed	0.4
Casein hydrolysate, for oral administration, unhydrolysed	0

* Except where mentioned, all materials were hydrolysed with conc. HCl for 3 hr. at 37° and tested at three or more levels.

† Values denote activity not strictly comparable to the streptogenin standard.

With the modified medium (Table 1), a linear dose response was regularly obtained when the logarithm of the dose was plotted against the dry weight of bacteria (Figs. 2 and 3). The agreement between triplicate tubes within an experiment at all the levels tested was satisfactory, with a coefficient of variation less than 5%.

The method has been applied to the assay of the streptogenin activity in a number of highly purified proteins and biological preparations (Tables 2 and 3). The results were expressed as a percentage of the activity of the standard (IA 37°). Since the method

of hydrolysis used for preparing the strepogenin standard was shown to liberate the maximum activity from the intact insulin molecule, the same procedure was adopted for the test materials. It is realized, however, that the activities may not be a true measure of the strepogenin content of materials, but represent only the activity liberated from proteins under comparable conditions. It may be that other methods of hydrolysis would yield higher values.

The materials were assayed usually at three or four levels. From the results, a logarithmic dose response curve was constructed and the relative potency was calculated from the linear dose response of the standard (IA 37°). If the slope of the dose response curve of the substance differed from that of the standard, the calculated activity varied at different levels. This is denoted in the tables by an asterisk and will be discussed below in more detail.

Insulin and liver preparations. The strepogenin activity of hydrolysed bovine insulins from different batches did not vary more than about 10%. Recrystallization of insulin or chymotrypsin digestion did not result in a further increase in potency. Unhydrolysed bovine insulin showed only negligible activity.

Sutherland, Cori, Haynes & Olsen (1949) have reported the separation of a hyperglycaemic-glycogenolytic (H-G) factor from amorphous and crystalline insulins. Insulin, Novo, which contains no H-G factor, had 90% of the strepogenin activity of the crystalline insulin standard. This indicated that the activity of the latter was not associated with the H-G factor.

The relative distribution of strepogenin activity in insulins showed certain species differences. The pig and sheep insulins were about four times as active as bovine insulin, but the greater activity could not be correlated with the differences in their amino-acid content (Sanger, 1949).

Wilson liver fraction L, and proteolysed whole liver powder, Hepamino Evans, tested up to levels of 50 and 4 mg. respectively, per 10 ml. medium, had a low activity (Table 2). At higher concentrations, however, their growth-stimulatory effect surpassed that of the strepogenin standard (Fig. 4).

Crystalline enzymes, viruses and other proteins (Table 3). Recrystallized pepsin and chymotrypsin 'Armour' when hydrolysed had a high activity of the order of 153–160%. Less pure samples were less active. In agreement with this finding, the peptides '16P', impurities obtained on purification of pepsin (Ingram, 1950), had also a negligible strepogenin content. The unhydrolysed enzymes showed very little activity (6–19%).

Turnip yellow mosaic virus and tobacco mosaic virus showed a high strepogenin content (56–86%) when hydrolysed, although the slopes of the dose response curves were different from that of the

standard. The unhydrolysed tobacco mosaic virus was only 6% active.

Myosin, lactoglobulin and plasma albumin were a good source of strepogenin activity, but the steeper response curves obtained with myosin and

Fig. 4. Growth responses of liver preparations compared with the strepogenin standard, IA 37°.

lactoglobulin were not strictly comparable to the strepogenin standard. Other proteins, including casein, had a negligible potency.

DISCUSSION

In the present study a medium has been used which is fully supplied with known growth factors and any increase in the rate of growth should be due to unknown factors. In the presence of an optimum concentration of the strepogenin standard an increase in growth of as much as about 1000 µg. of dry weight of bacteria per 10 ml. medium was obtained within 18–20 hr. of incubation.

Under these conditions the strepogenin activity seemed to be a specific property of certain partially hydrolysed proteins and could not be replaced by the various substances tested such as glutamine, glutamic acid, glutathione, asparagine or aspartic acid.

Intact protein molecules such as insulin, pepsin and tobacco mosaic virus showed little or no activity. In keeping with its supposed peptide structure the strepogenin effect was only observed when the protein molecule was broken down by mild acid hydrolysis or chymotrypsin. In agreement with Sprince & Woolley (1945) the strepogenin activity was lost on prolonged treatment with HCl.

The results for the various proteins and biological materials could be classified into two groups. The first showed a growth response curve lying, within experimental error, parallel with that of the strepogenin standard (Fig. 2). In the second group a steeper dose response curve was obtained, which diverged significantly from the slope of the standard

(Fig. 3). It seems that in these cases factors other than those present in acid-hydrolysed insulin were effective. The highest potency compared to insulin was found in enzyme and virus proteins, myosin, lactoglobulin and plasma albumin. Casein had a low activity. The comparison of our data with those of other authors (Sprince & Woolley, 1945; Wright *et al.* 1950) is difficult because of the different techniques employed. The strepogenin activities of trypsin, casein, haemoglobin, gelatin and salmin agree with those found by Sprince & Woolley (1945).

Of the biological materials assayed the liver preparations showed growth effects characteristic of the second group which differed in its behaviour from that of the insulin preparation. At low concentrations they showed little activity but at high levels the responses surpassed the maximum obtained with the strepogenin standard. Their activity remained the same whether the liver fractions were hydrolysed or not. It seems that besides strepogenin, other factors are present in liver preparations which are considerably more potent in stimulating the growth of the organism. The effects may be similar to those reported by Stokes, Koditschek, Rickes & Wood (1949) and Verdier & Ågren (1948).

The factors seem to be different from vitamin B₁₂ which has no stimulatory effect on *Lb. casei*. Moreover, the Wilson liver fraction L was active, although it does not contain the anti-pernicious anaemia factor.

The identical activity of unhydrolysed and hydrolysed liver preparations would seem to exclude factors of peptide nature, unless such peptides are normally present in the unhydrolysed materials, as indeed has been shown by Borsook *et al.* (1949), who isolated a peptide 'A' from unhydrolysed homogenates of liver.

The stimulatory effect of strepogenin preparations, observed during the early growth period, is most likely due to the shortening of the lag period. Whether the response given by the insulin prepara-

tion is due to one specific substance or a number of substances is difficult to decide. It seems that the stimulatory effect of partially hydrolysed proteins is more specific than suggested by the unspecific peptide theory of Dunn & McClure (1950). This would not explain the great differences in activities of proteins otherwise closely related. On the other hand, these activities cannot be explained by the existence of a single factor, but indicate that the strepogenin effect may be due to certain peptides which have a particular configuration.

SUMMARY

1. A satisfactory procedure for the assay of strepogenin activity has been developed.

2. The method was applied to the assay of strepogenin in highly purified proteins. Insulins from various species differed in their activity. Liver preparations, as well as some of the pure proteins assayed, seem to contain a factor or factors considerably more potent than the insulin preparation used as the strepogenin standard.

3. The peptide(s) responsible for the strepogenin activity were shown to be an integral part of the protein molecule and not associated with an impurity such as the hyperglycaemic-glycogenolytic factor present in commercial preparations of crystalline insulins.

4. Glutamine, asparagine and aspartic acid showed a slight strepogenin-like activity; glutamic acid and glutathione were inactive. None of these substances could replace the strepogenin effect of partially hydrolysed proteins.

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