ACTA CHEMICA SCANDINAVICA 9 (1955) 365—374

Study of a Peptic Degradation Product of Cytochrome c

II. Investigation of the Linkage between Peptide Moiety and Prosthetic Group

SVEN PALÉUS, ANDERS EHRENBERG and HANS TUPPY

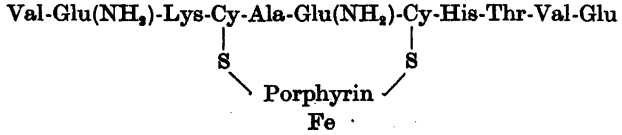
Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden and Zweites Chemisches Universitätslaboratorium, Vienna, Austria

The ferriporphyrin c-peptide obtained by peptic digestion of cytochrome c has been subjected to titrimetric, spectrophotometric and magnetic measurements. The results are compared with corresponding data for cytochrome c. In contradistinction to its parent protein, ferriporphyrin c-peptide is not altered spectrophotometrically by chloride ions at low pH values. Whereas the acid compound of ferricytochrome is transformed to the neutral form by a divalent dissociation with a pK of 2.12, it was found that the corresponding transformation of the peptide takes place in two separate steps with a pK of 3.4 and 5.8, respectively. The iron of the acid form has a susceptibility of 11 900 $\times$ 10^{-6} cgs emu and hence essentially ionic bonds. The alkaline form is covalent with a susceptibility of 2 100 $\times$ 10^{-6} cgs emu. The intermediate form (present between pH 3.4 and 5.8) has a tentative susceptibility of 8 800 $\times$ 10^{-6} cgs emu, and its structure is uncertain. The experimental data are related to structural differences between the peptide and cytochrome c, and the nature of the heme-linked groups is discussed. At pH 4.9 the ferriporphyrin c-peptide exhibits a peroxidatic activity more than 20 times stronger than that of cytochrome c.

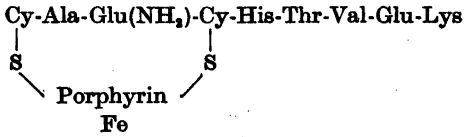
Attempts to understand the biological activity of a protein must be firmly based on considerations of the chemical structure of the molecule. Since many heme proteins have identical prosthetic groups [1], the activity associated with a particular heme protein must be a reflection of specific relationships existing between the prosthetic group and the protein moiety. Theorell and coworkers have for many years been concerned with the chemistry of heme proteins including cytochrome c. In 1939, Theorell [2] presented evidence suggesting two stable thioether bridges between the heme prosthetic group and the cytochrome c-protein. Data accumulated since that time strongly indicate the existence in fact of such bridges. In 1941, on the basis of titrimetric, spectrophotometric, and magnetic investigations, Theorell and Åkeson [3] proposed, in addition, two linkages between the iron atom of the prosthe-

tic group and imidazole groups of histidine residues in the protein moiety. Further investigations have substantiated this concept, and the nature of the iron-nitrogen linkages at various pH's has been clarified [4,5].

With the development of convenient methods for the preparation of relatively large amounts of pure cytochrome c[6] it has now become possible to study in greater detail certain structural aspects of the cytochrome c-protein itself. By controlled digestion with proteolytic enzymes [7], fragments of the protein firmly bound to the prosthetic group have been obtained. In the foregoing paper [8] the purification and structure of a peptic degradation product has been described:



Tuppy and Bodo [9] have also studied the structure of a tryptic degradation product:



The two fragments have certain important structural features in common. The porphyrin is bound *via* thioether bridges to two cysteine residues. In addition, a histidine residue is attached to one of the cysteine residues in the polypeptide sequence. The imidazole group of this histidine has been suggested by one of us to be one of the groups linked to the iron atom of the heme in the intact cytochrome c molecule [9].

This paper is concerned with an investigation of the peptic degradation product using the same physico-chemical methods which have been applied to the intact cytochrome c molecule.

MATERIAL AND METHODS

All the experiments were performed with the peptic degradation product of *cow* cytochrome c described in the foregoing paper [8]. The product is easily autoxidizable and is therefore obtained as a *ferriporphyrin* c-peptide. The iron content was determined by a modification of the method given by Lorber [10]. It was found to be 2.85 % in most of the preparations. All molarities are calculated on iron basis.

The titration apparatus described by Paléus was used [5]. N_2O or N_2 was passed through the titration vessel in order to diminish CO_2 errors. The pH of the solution was measured with a Radiometer Model 22 pH meter. The spectrophotometric experiments were carried out at 20° in a Beckman Model DU spectrophotometer. The absorption data are given as $\beta = d^{-1} \cdot c^{-1} \cdot \ln I_0/I$ cm²/mole, where natural logarithms are used, d is the optical path length in cm and c is the concentration in moles/cm³.

Especially when containing higher concentrations of salt and in certain pH regions the absorption values of the peptide solutions showed a tendency to decrease with time in an irreproducible manner. This troublesome behaviour of the peptide was eliminated to a large extent by carefully washing all vessels and absorption cuvettes with dilute ammonia and distilled water, and by rinsing them several times with the solution to be investigated.

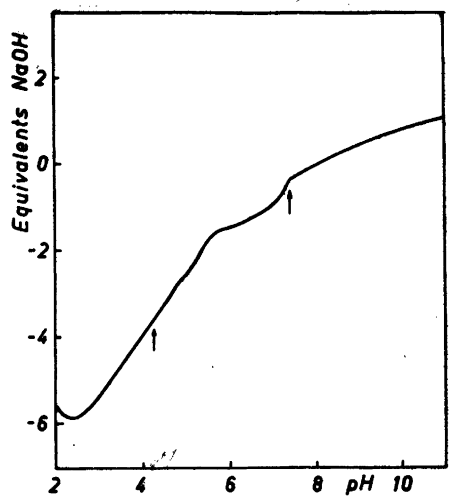


Fig. 1. Titration curve of the peptic degradation product of cow cytochrome c. The two arrows indicate the range of pH where the peptide precipitated.

The magnetic determinations were made in the apparatus constructed by Theorell and Ehrenberg[11]. A dilute solution of nickel chloride was used as a paramagnetic standard, $\chi_M = 4\,434 \times 10^{-6}$ cgs emu at 20° C, at which temperature all the measurements were made.

EXPERIMENTS AND RESULTS

Titrations

Approximately 3.5 mg of the peptide were suspended in 2.5 ml of water and dissolved by the addition of 1 *N* H_2SO_4 to obtain pH 2. The sample was then titrated with 1 *N* KOH. Under these conditions the peptide regularly precipitated at a pH of about 4.3 and did not redissolve until about pH 7.4. In this region the pH values, as measured immediately after each addition of alkali, were unstable but attained a constant value after longer waiting.

Several titrations were made with the same peptide preparation. It appeared difficult to reproduce the titration curve exactly, especially at the extreme pH values. One titration curve, corrected for blank, is shown in Fig. 1. The main shape of that curve was constantly reproduced and all the experiments indicated that about 7 equivalents were titrated between pH 2 and 11. This is in agreement with the structure of the peptide which has 7 titratable groups. Using the following tentative p*K* values: 3.1 and 4.4 for the carboxyls of glutamic acid, 4.6 for the two propionic acid residues, 6.3 for the imidazole of the histidine, 7.6 for the α-amino group of valine and 9.6 for the ε-amino group of lysine, a theoretical titration curve could be constructed which agreed nicely with the experimental observations except for the S-shaped part in the pH region 6—7.5. It has been suggested that the p*K* of the propionic acid residues in cytochrome c is about 5.5[2,5]. If this value is applied in our case, the p*K* of histidine must be shifted down to as far as 3.5, in order to obtain the same degree

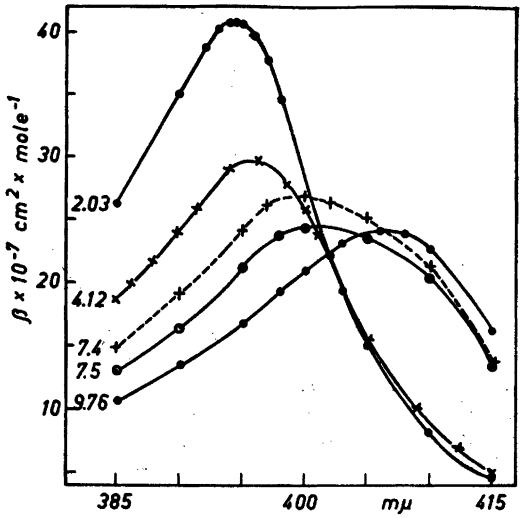


Fig. 2. Soret band of the peptide at different pH values. The pH is indicated at the beginning of every curve. The drawn curves are made from an acid stock solution, the dotted curve is from an alkaline stock solution. Cf text.

of coincidence with the experimental curve. The S-shaped part around neutrality remains unexplained, but might be a result of the precipitation and redissolution of the peptide.

Spectrophotometric investigations

The insolubility of the peptide made it difficult to obtain spectrophotometric data in the visible range of the spectrum. All the measurements were therefore made in the Soret band region.

a. The effect of the chloride ion concentration on the absorption spectrum in the Soret band region.

In order to determine the influence of anions on the absorption spectrum, aliquots of a 5 μM solution of the ferriporphyrin c-peptide in 0.001 N HCl (pH 3.03) were diluted with equal volumes of 0.001 N HCl containing varying concentrations of NaCl. In contrast to the findings of Boeri, Ehrenberg, Paul and Theorell [4] with intact cytochrome c, the position of the absorption maximum of the ferriporphyrin c-peptide at 395 mμ was not influenced by the Cl^- concentration. Furthermore, as shown in Fig. 4, the extinction at this wave length was not influenced by Cl^- concentrations between 0.001 and 0.3 M. At higher Cl^--concentrations a moderate decrease in absorption was observed. The same results were obtained in experiments at pH 2.00.

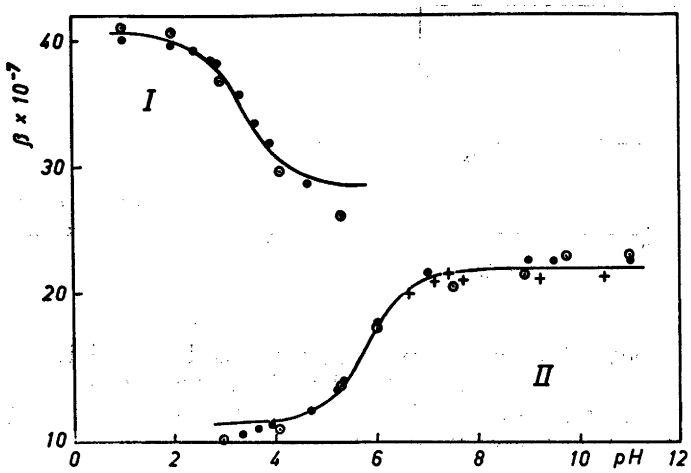


Fig. 3. Extinctions at 395 mμ (curve I) and 410 mμ (curve II) plotted versus pH. The different points are from different sets of experiments.

b. The effect of hydrogen ion concentration on the absorption spectrum in the Soret band region.

A 2.5 μM solution of peptide (2.85 % Fe) in 0.01 M HCl was prepared. To aliquots of this stock solution, either 3 N HCl or 1 N KOH was added from an "Agla" micrometer syringe, until the desired pH was obtained. Fig. 2 shows that in the strongly acid range the Soret band has a high maximum at 395 mμ. As the pH was increased towards 5.0, the extinction at 395 mμ decreased conspicuously, while the absorption maximum shifted only slightly towards the visible region. As the pH was increased above pH 5, however, the absorption maximum shifted markedly towards longer wavelengths, whereas the extinction changed only slightly.

Another set of curves were measured starting with a stock solution, 6.51 μM peptide (new preparation: 2.76 % iron) and 10^{-4} N NaOH. Aliquots of this stock solution were mixed with the double volume of acid, water or mixtures of water and NaOH to give different pH-values. At the most acid or alkaline pH's the new curves are almost identical with the corresponding ones of Fig. 2. One curve with an intermediate pH is included in that figure. It clearly shows the difficulty to obtain coinciding curves in this pH region where, however, the measured pH values of these unbuffered solutions are very unreliable.

In Fig. 3 the absorption values at 395 mμ and 410 mμ are plotted *versus* pH. The data at 395 mμ between pH 1 and 4.5 fit satisfactorily with a normal dissociation curve I with $n = 1$, $pK = 3.4$ and the asymptotic levels of $\beta_{395} = 40.5 \times 10^7$ cm²/mole at low pH and 28.5×10^7 cm²/mole at high pH. Also the data at 410 mμ are in fair agreement with a normal dissociation curve II: $n = 1$, $pK = 5.8$, $\beta_{410} = 9.5 \times 10^7$ cm²/mole and 21.9×10^7 cm²/mole at low and high pH, respectively.

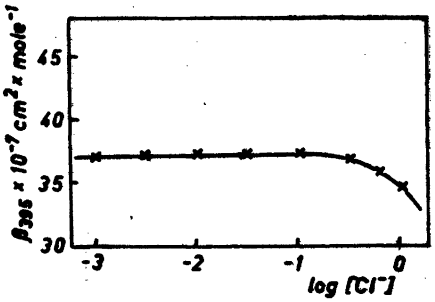


Fig. 4. The extinction at 395 mμ as a function of [Cl⁻] at pH 3.0.

From the spectrophotometric experiments just described it is clear that three forms of the polypeptide can be identified, one acid, one alkaline and one intermediate form. Because of the proximity between the two pK values the latter form is never present to 100 %.

Magnetic experiments

The following experiments were undertaken to determine the paramagnetic susceptibility of the three forms of the polypeptide described above.

About 2 mg of the dry polypeptide were accurately weighed in a small glass beaker, 1 ml of water was added and the sample was acidified by slow addition of 3 *N* HCl from a micrometer syringe, during rapid stirring by means of a magnetic stirrer. The pH was measured with a carefully cleaned and dried glass electrode and adjusted to about 1.5. The sample was immediately transferred from the open beaker to a closed tube. A large amount of a buffer with the same proportions of water and acid was prepared and measured as blank in the magnet. After that the sample was measured and then brought back to the closed tube. The blank was read again.

Largest possible amount was accurately pipetted over to the cleaned beaker and the pH of that sample was adjusted to between 3.7 and 4.0 by 3 *N* NaOH added from a micrometer syringe. Higher pH values must be avoided because of the risk of precipitation. The sample was transferred to a closed tube, a proper blank buffer was prepared from the old buffer and the magnetic measurements made.

Table 1. Paramagnetic susceptibilites of the iron of the ferri porphyrin peptide at different pH values in the presence of chloride or sulfate ions.

HCl			H_2SO_4		
pH	c_{Cl^-} m*M*	$\chi_{Fe} \times 10^6$ cgs emu	pH	$c_{SO_4^{2-}}$ mM	$\chi_{Fe} \times 10^6$ cgs emu
1.50	37	11 360	1.50	32	11 960
3.70	37	9 600	3.90	31	9 550
10.60	37	1 930	10.90	31	2 230
1.50	68	12 750	1.40	91	11 420

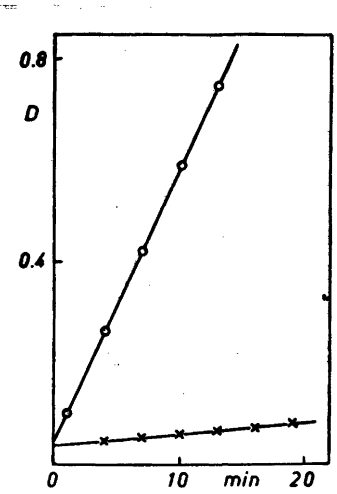


Fig. 5. The peroxidatic activity of the peptide (o) and cytochrome c (×).

A measurement was similarly made at pH 10.5—11.0 and finally the sample was again brought to pH 1.5 and remeasured. An iron determination was made on the rest of the sample.

The same procedure was repeated on a new sample but with hydrosulfuric acid instead of hydrochloric acid.

From the iron content of the dry peptide and the dilutions made, it is easy to calculate the concentration of iron in every magnetic determination. In both sets of experiments, however, the direct determination of iron in the sample after the last measurement in the magnet showed 4 % higher iron content than calculated. This is probably due to evaporation and an additive correction of 1 % per step in the treatment of the sample was introduced.

Because of the high iron content of the polypeptide the diamagnetic contribution is of comparatively small importance. A rough value for it, 100×10^{-6} cgs emu, was obtained by analogy with known figures of various heme proteins studied in this laboratory.

The susceptibilities determined are listed in Table 1. No definite influence by the anions can be seen at the low pH. According to the spectrophotometric investigation the acid form is present to practically 100 % at pH 1.5 and the alkaline form at pH above 10.5. We thus obtain $\chi_{acid} = 11\,900 \times 10^{-6}$ and $\chi_{alkaline} = 2\,100 \times 10^{-6}$ cgs emu. From these values, the data at pH 3.70 and 3.90 and the two pK values of 3.4 and 5.8, we calculate $\chi = 8\,800 \times 10^{-6}$ cgs emu as a tentative value of the susceptibility of the intermediate form.

Peroxidatic activity

The procedure designed by Paul and Avi-Dor [12] for the assay of horseradish peroxidase, in which mesidine serves as hydrogen donor, was used in a simplified form to compare the known slight peroxidatic activity of cytochrome c with that of its peptic degradation product.

Two flasks, each containing 35 ml 0.1 *M* acetate buffer pH 4.9, 3 ml of an aqueous 0.2 *M* mesidine hydrochloride solution, and 2 ml 0.1 *M* hydrogen peroxide, were cooled down to 0° in a bath containing crushed ice. Then 1.0 ml of 0.2 m*M* solutions of bovine cytochrome c (0.433 % Fe) and of the ferriporphyrin c-peptide in 0.05 *M* NH_3 were added. From the reaction mixtures kept at 0°, after different time intervals, aliquots were taken out and their extinctions at 490 mμ measured immediately in a Beckman spectrophotometer. In Fig. 5 the extinction readings are plotted *versus* time. The slope of the straight lines obtained is proportional to peroxidatic activity.

DISCUSSION

As a consequence of their investigations, Theorell and coworkers have suggested a conceptual picture of cytochrome c which reflects its observed properties. The prosthetic group can be imagined to be a hematin disc embedded in a crevice of the protein moiety. The iron in the prosthetic group is coordinated to two haemochromeforming groups in addition to the four porphyrin nitrogen atoms. Presumably because of the structural architecture, the iron atom is inaccessible to molecular oxygen and is therefore non-autoxidizable. At the same time, electron transfer is made possible.

At acid reactions, however, the covalent bonds between the iron and the hemochrome forming groups of ferri-cytochrome c are ruptured (p*K* 2.12) [4], and the bonds to the iron assume an essentially ionic character. Chloride ions can enter yielding a dichloride derivative of the acid ferri-cytochrome c which has a peculiar structure around the iron atom with covalent bonds to the four pyrrolic nitrogens and two ionic bonds perpendicular to the heme disc (Boeri, Ehrenberg, Paul and Theorell [4]). The unusual stability of this structure, and the fact that even at low pH values, the protein moiety and prosthetic group do not dissociate are presumably due to the stable thioether bridges.

This investigation reveals that there are some interesting similarities and dissimilarities between the native cytochrome c and its peptic degradation product.

The spectrum of the ferriporphyrin peptide in the Soret band region is at very low pH values quite similar to that of acid ferricytochrome c ("cyt-$2H^+$", Boeri *et al.*[4]). The absorption maximum of both compounds is situated at 395 mμ; the absorption coefficient ($\beta \times 10^{-7}$ cm^2/mole) is about 41 for the peptide whereas Boeri *et al.*, by extrapolation to $[Cl^-] = 0$, calculated it to be around 50 for "cyt - $2H^+$".

The magnetic susceptibility of the two compounds are not equal. For the peptide χ_{Fe} was measured to be 11 900 $\times 10^{-6}$ cgs emu, and the extrapolated value for "cyt - $2H^+$" was 16 200 $\times 10^{-6}$ cgs emu. Both values are, however, compatible with the presence of 5 odd electrons in the iron atom, and the bonds involved are essentially ionic. The difference in paramagnetic susceptibility might be reflected in heme-linked groups of different nature.

As shown here, contrary to the findings in the intact enzyme, the peptic degradation product of cytochrome c does not exhibit a "chloride effect", its spectrophotometric properties in the Soret band region remain unchanged on

increase of the chloride ion concentration up to a molarity of 0.3. The failure of chloride ion to be incorporated between iron and hemochrome forming groups of the peptide can be attributed to the lack of the firm architecture present in the undenatured cytochrome c molecule when the covalent bonds between them and the iron are broken.

An increase in pH leads to a simultaneous release of 2 protons from "Cyt $-2H^+$" (pK 2.12) and the formation of neutral cytochrome c. This behaviour has been interpreted as indicating that the two hemochrome-forming nitrogenous groups of the protein moiety of cytochrome c are identical, and are symmetrically attached and equally firmly bound to the iron. In the peptide investigated, only one histidine is present, and the protons are, as might be expected, not released simultaneously. The first is released at a pK of 3.4 and the second at 5.8.

No further spectral changes could be derived in the Soret band region at pH's below 11. The alkaline form of the peptide has its absorption maximum at 406 mμ with $\beta = 24.5 \times 10^{-7}$ cm^2/mole, which is nearly identical with the corresponding values of neutral cytochrome c, 407 mμ and $\beta = 26.0 \times 10^{-7}$ cm^2/mole. Also the paramagnetic susceptibilities are in close agreement, 2 080 (mean value) and 2 120 $\times 10^{-6}$ cgs emu, respectively. Our conclusion is that the iron in the alkaline form of the peptide is held by six octahedral covalent bonds. It is interesting to observe that the great similarity of paramagnetism and absorption properties in the Soret region do not correspond to identical groups coordinated to the iron. In cytochrome c two histidine residues are supposed to be bound but in the peptide only one histidine is present and could possibly be heme-linked.

The intermediate compound (appearing between pH 3.4 and 5.8) of the peptic degradation product has its Soret maximum at about 396 mμ with $\beta \sim 30 \times 10^{-7}$ cm^2/mole. The paramagnetic susceptibility of this compound is found to be about 8 800 $\times 10^{-6}$ cgs emu. In this case no close analogy could be drawn with any compound of native cytochrome c. It is true that the ferricytochrome dichloride (cyt. $- 2H^+ - 2Cl^-$) has a paramagnetism of comparable magnitude but it is somewhat lower, and its Soret band is situated at 401 mμ. Probably the most suitable assumption is nevertheless that the susceptibility 8 800 $\times 10^{-6}$ cgs emu is due to a structure with 3 odd electrons in the iron atom as in the ferricytochrome dichloride. This type of compound is rare and the types of bonds involved are but little known. The possibility of 5 odd electrons and essentially ionic bonds cannot, however, be totally excluded. In any case the transition with pK = 3.4 means that one heme-linked group is titrated and that the bonds to the iron are more or less profoundly changed.

The present investigations do not give any unambiguous indication of the nature of the heme-linked groups of the ferriporphyrin c-peptide. The structure of the peptide, however, limits the number of groups which can be involved. One of us (Tuppy [9]) has suggested that the histidine residue is heme-linked. From the titration curve it cannot be decided whether this group has a pK around 3.5 or 6.3. A heme-linked histidine group would be expected to have a pK lower than the "normal" value (5.5—8.0) but higher than that of ferricytochrome c (pK = 2.12) [4]. The pK of 3.4 is within that range. On the other hand it has been proposed that the imino groups of the two heme-linked

imidazoles in cytochrome c are titrated with pK's of about 9.5 [3]. The titration curve of the peptide shows that the imino group in this case cannot be titrated below pH 11. This means that either the imidazole group is heme-linked and the pK of its imino group is above 11 or the imidazole group is not heme-linked. The former possibility is in reasonable agreement with the experimental data if it is assumed that the amino group of either lysine or valine is also heme-linked with the pK shifted to 5.8. Such a shift would not distort the theoretical titration curve too much.

It is *a priori* also possible that an ionically bound carboxyl group is titrated with a pK of 3.4. The pK of 5.8 would then correspond to the titration of the imidazole or one of the amino groups. The "acidifying" effect of the heme linkage on the imidazole might be so much smaller in the peptide compared to cytochrome c as to justify the assumption that it is titrated at the lower limit of its normal region. The carboxyl group, however, does not necessarily need to be linked to the iron in the alkaline form as it could be competitively replaced by the already titrated amino group of valine without changing the titration curve. The possibility that one of the carboxyl groups of the C-terminal glutamic acid is heme-linked in the way just discussed seems much less likely than the former assumption that the imidazole of histidine and the ε-amino group of lysine or the α-amino group of valine are the heme-linked groups involved.

The configuration around the heme of the peptide might be somewhat similar to that of peroxidase. On account of this assumption the activity experiment was undertaken. It showed that the peroxidatic activity of the peptide is more than 20 times stronger than that of cytochrome c and thus about one per cent of the activity of peroxidase itself [12].

The authors want to express their sincere gratitude to Professor H. Theorell and to Professor F. Wessely for advice, encouragement and criticism during the work. We also want to thank Laborator K. G. Paul and Dr. W. J. Rutter for valuable discussions and Miss B. Rolander for careful and valuable assistance.

The work was supported by grants from *Statens Naturvetenskapliga Forskningsråd* for two of us (A. E. and H. T.) and from *Medicinska Prisgruppens särskilda fond* and *Medicinska Forskningsanslaget* for one of us (S. P.).

REFERENCES

1. Theorell, H. *Advances in Enzymol.* **7** (1947) 265.
2. Theorell, H. *Biochem. Z.* **298** (1939) 242.
3. Theorell, H. and Åkeson, Å. *J. Am. Chem. Soc.* **63** (1941) 1804.
4. Boeri, E., Ehrenberg, A., Paul, K. G. and Theorell, H. *Biochim. et Biophys. Acta* **12** (1953) 273.
5. Paléus, S. *Acta Chem. Scand.* **8** (1954) 971.
6. Paléus, S. and Neilands, J. B. *Acta Chem. Scand.* **4** (1950) 1024.
7. Tsou, C. L. *Biochem. J. (London)* **49** (1951) 362.
8. Tuppy, H. and Paléus, S. *Acta Chem. Scand.* **9** (1955) 353.
9. Tuppy, H. and Bodo, G. *Monatsh.* **85** (1954) 807, 1024.
10. Paul, K. G. *Acta Chem. Scand.* **2** (1948) 430.
11. Theorell, H. and Ehrenberg, A. *Arkiv Fysik* **3** (1951) 299.
12. Paul, K. G. and Avi-Dor, J. *Acta Chem. Scand.* **8** (1954) 649.

Received January 4, 1955.