ACTA CHEMICA SCANDINAVICA 20 (1966) 1487–1496

On the Heterogeneity of Beef Heart Cytochrome c

III. A Kinetic Study of the Non-enzymic Deamidation of the Main Subfractions (Cy I — Cy III)

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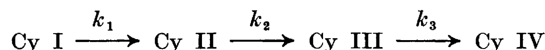
The stability of subfractions Cy I—Cy IV was studied, and the following results were obtained:

(1) At pH 7, all subfractions are quite stable when stored at 4°C and −20°C or when subjected to repeated freezing and thawing.

(2) Subfraction Cy I was converted to Cy II—Cy IV when exposed to buffers of appropriate pH's and temperatures. The conversions proceed as consecutive reactions in the following sequence,



$$\text{Cy I} \xrightarrow{k_1} \text{Cy II} \xrightarrow{k_2} \text{Cy III} \xrightarrow{k_3} \text{Cy IV}$$

and in no case has any reversibility been observed. These reactions proceed more rapidly at higher ionic strength and temperatures. At 37°C, the pseudo first-order rate constants k_1 and k_2 revealed minimum values at about pH 5, and they increased at increased concentrations of protons or hydroxyl ions. However, at 4°C, no measurable rates were observed at $3 < \text{pH} < 9.3$ (formate and borate buffers, $\mu \approx 0.1$).

(3) A rapid conversion of subfraction Cy I to Cy II—Cy IV was observed under the conditions commonly used for crystallization of cyt. c by ammonium sulphate.

(4) The conversions are accompanied by the liberation of ammonia at a rate and in a stoichiometric amount that is compatible with the hydrolysis of one amide group in each conversion step. The nature of the labile amide groups and the difference in the calculated rate constants k_1 and k_2 as a function of pH are discussed.

(5) The *in vivo* origin of the subfractions Cy II—Cy IV is dicussed in view of these results.

It has been established that monomeric beef heart cytochrome *c** can be separated into four subfractions (termed, in decreasing order of mobility towards the cathode at neutral pH, Cy I, Cy II, Cy III, and Cy IV) by means of disc electrophoresis on polyacrylamide gel [1] and moving-boundary electro-

* The following abbreviation will be used: Cyt. c = cytochrome c.

phoresis.[2] The difference in electrophoretic mobility between Cy I and Cy II can be explained by the demonstrated difference of one amide group,[2] *i.e.* Cy II contains one free carboxyl group more than Cy I. The difference in mobility between Cy II and Cy III can also be explained by a difference in the content of amide (free carboxyl) groups,[2] though no chemical analyses have been carried out on Cy III so far.

In the first paper of this series [1] some preliminary investigations into the interconvertibility of the subfractions were performed. Thus, prolonged storage of the minced muscle before extraction resulted in a higher percentage of the minor subfractions (Cy II—Cy IV),[1] and the possibility of interconversion as the origin of these subfractions was suggested. However, no conclusive evidence could be given at that time. Furthermore, repeated runs of unresolved cyt. c [2] at increasing alkaline pH values in the moving-boundary electrophoresis resulted in a gradual increase in the percentage of the minor subfractions indicating the possibility of a conversion of Cy I to the other fractions. Thus, in order to obtain a clearer idea of these phenomena, the stability of Cy I under some extreme environmental conditions was studied in more detail. The present paper describes the kinetics of the non-enzymic conversion of Cy I into Cy II—Cy IV as a function of pH, ionic strength and temperature.

MATERIALS AND METHODS

Cytochrome c, extracted from beef heart muscle by dilute sulphuric acid at pH 4, was obtained in the monomeric form by gel filtration on Sephadex G-75,[3] and the subfractions Cy I—Cy III were isolated by preparative disc electrophoresis on polyacrylamide gel as previously described.[1] Each fraction eluted from the acrylamide gel was re-run in an analytical column with the same technique to ensure that no contamination had occurred while cutting the gel.

L-*Asparagine* was of the highest analytical purity (from Schwarz Laboratories, Inc., N.Y., U.S.A.).

The *concentration of cyt. c* was assayed as previously described;[3] the specific extinction coefficient $E_{1\,\mathrm{cm}}^{1\,\%}$ at 550 mμ red = 23.94 was used.

Electrophoresis. Analytical disc electrophoresis on polyacrylamide gel was carried out as previously described.[1] High-voltage paper electrophoresis was carried out as described by Åkeson and Theorell,[4] and the paper was sprayed for amino acids and peptides with a 0.3 % (w/v) solution of ninhydrin in 95 % (v/v) of ethanol.

Conversion of the main subfraction (Cy I) at extreme pH values. The effect of pH on the stability of Cy I was estimated in formate and borate buffers (pH-range 2.7 to 11.5). Incubation mixtures were prepared in the following manner: The buffer and a solution of cyt. c in 1 mM ammonia were mixed (1:1, v/v) to give a total volume of 1 ml and an initial concentration of Cy I in the mixtures of about 200 μM; except where otherwise stated the final ionic strength was 0.1. The mixtures were filtered through a Millipore filter, type GS (0.22 μ) and incubated at different temperatures (see results). At the end of the incubation period, the samples were immediately cooled in melting ice and adjusted to pH 7.0 by means of 0.01 N HCl (NaOH), and dialyzed against 20 mM ammonium phosphate buffer of pH 6.9. Finally, the samples were subjected to disc electrophoresis, and the relative percentages of the individual components estimated.[1]

Deamidation of asparagine. L-Asparagine was exposed to different pH values according to the procedure described above for cyt. c; the initial concentration of L-asparagine was 10 mM. At the end of the incubation period, the samples were immediately frozen at −80°C (dry ice-acetone) and stored at −20°C. The content of asparagine and aspartic acid was assayed by column chromatography according to the method of Spackman

et al.[5] The pseudo first-order rate constant (k (sec^{-1})) for the hydrolysis of asparagine was calculated from the proportion of amide to acid when determined as a function of time.

Liberation of ammonia from subfraction Cy I at pH 11.6 as a function of time was determined by the Conway microdiffusion technique.[2,6] The hemoprotein, pretreated as previously described for amino acid and amide analyses,[2] was mixed with saturated potassium tetraborate in 10 % (w/v) KOH (1:2, v/v) in the outer chamber of the Conway vessel.

pH-measurements. A glass electrode pH-meter — Radiometer, Copenhagen, Model 25 SE — was used. It was standardized against Beckman standard buffers (pH = 4.01 and pH = 9.22 at 25°C). The pH values of reaction solutions were determined at the temperatures reported in results.

RESULTS

In order to examine the stability of the various subfractions they were exposed to certain extreme environmental conditions and afterwards studied by means of disc electrophoresis on polyacrylamide gel. The ferric form[2] of the purified subfractions was used throughout.

Stability of electrophoretic pattern

(a) Effect of prolonged storage at 4°C and —20°C. The effect of prolonged storage on the electrophoretic behavior of Cy I, Cy II, and Cy III was studied. The cyt. c preparations were dialyzed against 40 mM ammonium phosphate buffer, pH 6.9, and samples of 1 ml were stored in sealed glass tubes at 4°C and —20°C. All subfractions moved as homogeneous fractions with their original mobilities even after having been stored for at least 4 months at either temperature.

(b) Effect of repeated freezing and thawing. The cyt. c solutions were the same as in the above-mentioned experiments. Samples of 1 ml in 5 × 1 cm glass tubes were frozen (i) slowly, by placing the tubes in a deepfreeze (—20°C) or (ii) rapidly, by putting the tubes in dry ice-acetone (—80°C), and in either case were rapidly thawed by shaking them under running tap water (20°C) for 10—20 sec. However, even after freezing and thawing for 10 times, Cy I and Cy II moved as homogeneous fractions with their original mobilities.

Non-enzymic conversion of the subfractions Cy I—Cy III

For purposes of comparison, the reaction conditions chosen were primarily as similar as possible to those used in the moving-boundary electrophoresis experiments.[2] Thus, when stored at 4°C in borate buffer, $\mu \approx 0.1$ and pH $\gtrsim 9.3$, Cy I revealed a slow conversion to two components of lower electrophoretic mobility than Cy I (Fig. 1, c).

(a) Characterization of products of conversion. The two new components formed by this conversion revealed electrophoretic mobilities identical with those of Cy II and Cy III. Their identity was confirmed by the fact that single zones were obtained in the electropherogram when the individual zones were isolated from the polyacrylamide gels, mixed with pure preparations of Cy II and Cy III, respectively, and run on separate columns. In addition, ninhydrin-

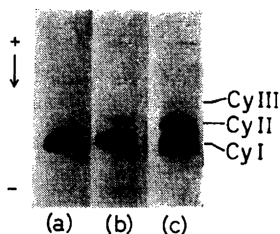


Fig. 1. Photograph of disc electrophoresis patterns showing the conversion of subfraction Cy I into Cy II and Cy III at 4°C. Borate buffer, pH (4°C) = 11.73 and $\mu \approx 0.1$. The electropherograms were obtained after an incubation period of 0 h (a), 68.5 h (b), and 166.5 h (c).

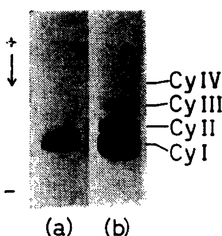


Fig. 2. Photograph of disc electrophoresis patterns showing the conversion of subfraction Cy I into Cy II—Cy IV at 37°C. Borate buffer, pH (37°C) = 10.4 and $\mu \approx 0.1$. The electropherograms were obtained after an incubation period of 0 h (a) and 54.6 h (b).

positive substances, except cyt. c and ammonia, could not be detected by high-voltage paper electrophoresis of the incubation mixtures.

(b) Progress curve, reaction order and calculation of rate constants. During the incubation period subfraction Cy II first appeared (Fig. 1, b) in the electropherogram and then finally Cy III (Fig. 1, c); Cy IV was revealed only when the conversion was carried out at a higher temperature, *e.g.* 37°C (Fig. 2, b). Furthermore, by conversion of the individual subfractions at 37°C, Cy II gave both Cy III and Cy IV, and Cy III gave only Cy IV. Thus, in no case has any reconversion, *e.g.* from Cy II to Cy I, been observed. Fig. 3 shows the progress curve for the conversion of Cy I to Cy II at pH 11.73, $\mu \approx 0.1$ and 4°C, and it is seen that the data fit in well with a first-order reaction; the pseudo first-order rate constant was calculated to be $k_1 = 3.9 \times 10^{-7}$ (sec^{-1}). In order to calculate the rates of the conversions of Cy I $\xrightarrow{k_1}$ Cy II and of Cy II $\xrightarrow{k_2}$ Cy III the common equations for two consecutive first-order reactions were applied; k_1 was obtained directly and k_2 graphically.

(c) Effect of temperature and pH. At 4°C no conversion was observed in the pH-range 3.0—9.3 following a reaction time of 8 days, but at pH > 9.3 the rate of conversion increased with increasing hydroxyl ion concentration (Fig. 4). On the other hand, at 37°C there was a measurable conversion at any pH value, but the rate of conversion increased by increasing the concentration of protons or hydroxyl ions; a minimum rate was observed at about pH 5 (Fig. 4).

(d) Effect of ionic strength. From Fig. 5 it is seen that the higher the ionic strength the more rapid was the rate of conversion.

(e) Liberation of ammonia. Ammonia was released during the conversion of Cy I to the other components (Cy II—Cy IV), as shown in Fig. 6. The time

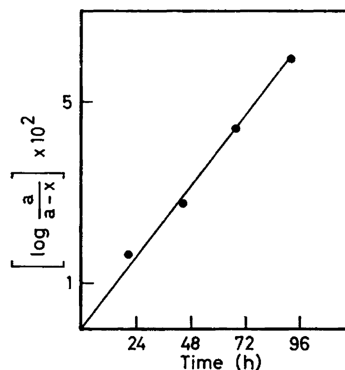


Fig. 3. Time course for the conversion of Cy I to Cy II in borate buffer, pH (4°C) = 11.73, $\mu \approx 0.1$; 4°C. a and x represent the initial concentration of Cy I and the amount of conversion products (Σ Cy (II—III)), respectively.

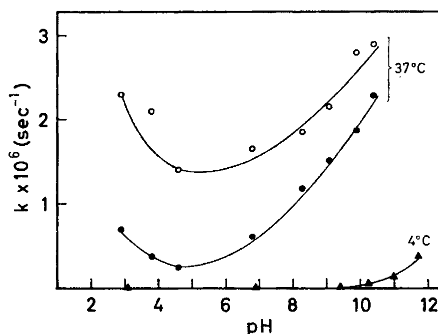


Fig. 4. Effect of pH and temperature on the first-order rate constant for the conversion of subfraction Cy I into Cy II (▲ and ●) and of Cy II into Cy III (○). Formate and borate buffers of ionic strength 0.1 were used. For experimental details see text.

progress curve revealed a bi-phasic course where the steep initial slope in the main part reflects the release of free ammonia in the preparation; 99.9 % of any free ammonia should be adsorbed in the acid of the inner chamber within 2.5 h under the experimental conditions used.[6] The second slope of the curve reflects the ammonia liberated following hydrolysis of labile amide groups in the polypeptide chain, and by extrapolation of this slope to zero time a more accurate estimate of the free ammonia was obtained (*i.e.* 0.18 μmole NH_3/μmole cyt. c). The observed value for ammonia liberated by hydrolysis of amide groups (0.98 μmole NH_3/μmole cyt. c) is in fairly good agreement with the amount expected (0.82 μmole NH_3/μmole cyt. c) by assuming a hydrolysis of one amide group in each conversion step.

(f) Effect of ammonium sulphate. The effect of ammonium sulphate on the stability of the subfractions is of particular interest since this salt has been widely used in the procedure for large scale purification as well as in the crystallization of cyt. c. Furthermore, it has been reported that yeast cyt. c becomes autoxidizable [7] and that lactoperoxidase reveals changes of the light absorption spectrum at the Soret band [8] in the presence of ammonium sulphate.

From the data given above one would expect that a rather rapid conversion of Cy I to Cy II—Cy IV could occur under the conditions used for crystalliza-

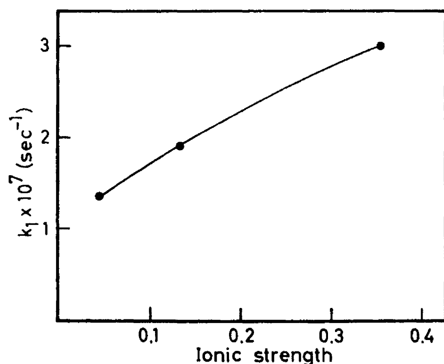


Fig. 5. Effect of ionic strength on the conversion of Cy I into Cy II in borate buffer of pH (4°C) = 11.14; 4°C. Symbols as in Fig. 3.

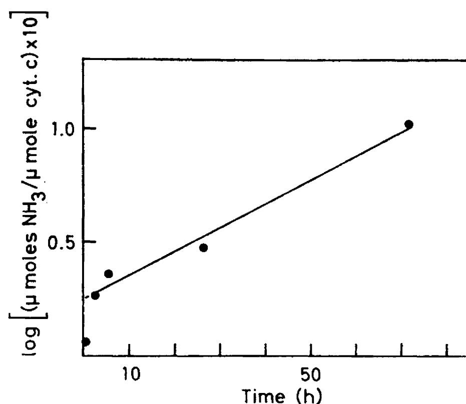


Fig. 6. Liberation of ammonia during the transformation of Cy I to the other subfractions as determined by the Conway microdiffusion technique. Standard unit; 1 ml N/1000 H_2SO_4 with indicator in the inner chamber and 1.5 ml cyt. c solution of pH 11.6 in the outer chamber. 23°C. The second slope of the bi-phasic curve was calculated by the least-square method which gave $y = 0.2553 + 0.01156\ x$. At $t = 72$ h, the percentage distribution of subfractions were: Cy I 37.0 %, Cy II 46.3 %, Cy III 14.0 %, and Cy IV 2.7 %.

tion of cyt. c, and that was actually the case. Thus, when ascorbic acid reduced Cy I was treated by ammonium sulphate of 80 % satn. at room temperature,[7] a rapid conversion to Cy II—Cy IV was observed even at neutral pH; the first-order rate constant for the conversion of Cy I → Cy II was calculated to be $k_1 = 5.7 \times 10^{-7}$ (sec^{-1}) at pH 10, and $k_1 = 4.4 \times 10^{-7}$ (sec^{-1}) at pH 4.

Deamidation of asparagine in vitro

The non-enzymic methods for the determination of glutamine and asparagine were previously based on the greater temperature lability of the amide group of glutamine as compared with that of asparagine.[8a] Thus, Vichery *et al.*[9] found that glutamine was hydrolyzed at a rate which was approx. 90 times greater than that of asparagine at pH 5.1 and 100°C. Furthermore, it is well documented that the stability of glutamine is very dependent on temperature,[9–11] but such data are not available for asparagine.

In Table 1 the rate of deamidation of L-asparagine obtained in the present study is compared with the data previously found for glutamine. It is seen that the latter amide is hydrolyzed at a rate which is 95—120 times greater than that of L-asparagine in the pH range 2.9—10.4.

DISCUSSION

The results presented above show that the small differences in net charge that serve to distinguish the subfractions Cy I—Cy III are based on rather stable properties, though evidence for conversion of Cy I to the other subfractions has been obtained. From a practical point of view it is important that all subfractions are quite stable (at least for a few months) when stored at −20°C and pH 7.

The strong tendency of Cy I to convert at extreme pH values, especially at higher temperatures, is not surprising in view of previous studies on different hormones. For some of these polypeptides hydrolysis of the amide groups of glutamine/asparagine residues has been clearly demonstrated either in alkaline solution (adrenocorticotropic hormone,[12,13] lactogenic hormone,[14] and growth hormone [14]) or in acid solution (insulin [15,16]). In all of these hormones the hydrolysis resulted in a change in their net charge which was revealed either by countercurrent distribution, by chromatography, or by electrophoresis. However, no detailed kinetic study of the conversion reaction, as reported in this paper for cyt. c, was carried out.

The conversion of subfraction Cy I to Cy II—Cy IV appears to proceed as consecutive reactions in the following sequence,

$$\text{Cy I} \xrightarrow{k_1} \text{Cy II} \xrightarrow{k_2} \text{Cy III} \xrightarrow{k_3} \text{Cy IV} \qquad (1)$$

and in no case has any reversibility been observed. Furthermore, it seems clear that these transformations are due to deamidation of accessible glutamine and/or asparagine residues of the hemoprotein, and the following facts may support the view that the labile amide groups belong to glutamine residues:

(1) It is well known that free glutamine is more easily deamidated than free asparagine at high temperatures, *e.g.* 100°C,[8a,9] and from Table 1 it is seen that this is also true at 37°C over a wide pH range. At pH 10.4, the rate of hydrolysis is about 100 times greater for L-glutamine (0.2 M phosphate buffer) than for L-asparagine (borate buffer, $\mu \approx 0.1$).

(2) To explain the heterogeneity of monomeric beef heart cyt. c on the basis of a difference in amide content, three labile or accessible amides need to be present in Cy I. From the amino acid sequence study of Yasunobu *et al.*[17] we know that unresolved beef heart cyt. c (*i.e.* mainly Cy I) contains three glutamine residues, *i.e.* in the positions 12, 16, and 42.

(3) The kinetics of the transformation of Cy I → Cy II and of Cy II → Cy III revealed a striking similarity to the non-enzymic deamidation of free glutamine.[9] Firstly, at 37°C the pH-dependence is very similar with minimum rates of conversion at about pH 5 (Table 1). Secondly, the rates of both reactions increase at higher ionic strength. Finally, from Table 1 it is seen that at 37°C the rate of the conversion of Cy I → Cy II (k_1) at pH 2.9 is only 7 times smaller than that reported for free glutamine in 0.2 M phosphate buffer, but as much as 17 times greater than that found for free asparagine; the rate of the conversion of Cy II → Cy III (k_2) was calculated to be even higher than k_1. The validity of the interpretation given above must, however, await further experimental evidence.

Table 1. The rate of conversion of Cy I to Cy II as compared to the non-enzymic deamidation of L-asparagine and glutamine at 37°C.

pH (37°C)	First-order rate constant (k (sec^{-1}) $\times$ 10^6)		
	Cy I → Cy II[a]	Deamidation of L-asparagine[a]	Deamidation of glutamine[b]
2.9	0.68	0.04	4.8
3.8	0.37	—	2.3
4.6	0.24	—	1.8
6.8	0.62	0.00	4.3
8.3	1.19	0.08	8.3
9.1	1.51	—	11.3
9.9	1.87	—	13.8
10.4	2.23	0.18	17.1

[a] Data obtained in formate buffer (acid pH) and borate buffer (alkaline pH), $\mu \approx 0.1$.
[b] Data, taken from Gilbert *et al.*, Ref. 10, were obtained in 0.2 M phosphate buffer. The rate of deamidation was stated to be approx. eight times lower in borate than in phosphate buffer.

Some glutamine-containing proteins and polypeptides act as substrates for guinea pig liver transglutaminase while others do not.[18–22] This difference in reactivity of polypeptide-bonded glutamines has been attributed to either (i) a difference in their position in the sequence (primary structure effect)[22] or (ii) to a different accessibility of their amide group arising from polypeptide structural configurations (secondary and tertiary structure effect).[21] It is reasonable to assume that both these factors are also relevant for the non-enzymic deamidation of glutamine residues in proteins, *e.g.* in cyt. c. Thus, the large temperature effect observed for the conversions indicates that the conformation of the protein is an important factor for the rate of the reactions. This view is in accordance with the optical rotatory dispersion studies of Urry [23] that ferri-cyt. c has its most native conformation at 4°C and pH 5—7; by increasing the concentration of protons or hydroxyl ions and/or the temperature there is an increasing degree of unfolding. It should also be noted that subfractions Cy II—Cy IV represent some conformational variants of the native form (Cy I) based on their different light absorption spectra [2] and their (Cy III and Cy IV) ability to bind the anion dye bromocresol green.[24] Thus, it appears from the data obtained so far that glutamine/asparagine residues may be important for the maintenance of the native conformation of the hemoprotein.

Based on the concept discussed above, statistically one should expect that Cy II is a mixture of isomers containing one of the 7 amide groups on different carboxyls. These variants should have the same isoionic point, but may nevertheless be resolvable if there is a difference in their interaction with, *e.g.*, buffer ions which is important for the surface charge of proteins. Furthermore, in acid solution the isomers may be resolved if the carboxyl groups in question have different pK values. In this connection it may, therefore,

be worth mentioning that subfraction Cy II, which appeared to be homogeneous on disc and moving-boundary electrophoresis, was resolved into two components by chromatography on Duolite CS-101.[1] Furthermore, recent studies by means of isoelectric fractionation by electrolysis in a natural pH-gradient[25] have confirmed this heterogeneity of Cy II for which the nomenclature Cy II_1 and Cy II_2 will be used. A third component might also be expected, but so far its presence has not been revealed.

The experiments described in this paper provide additional support of the view previously suggested that the subfractions Cy II—Cy IV of monomeric beef heart cyt. c arise by conversion of the main subfraction (Cy I) and that they all occur *in vivo*. Thus, at a physiological pH, ionic strength and temperature there is a reasonable rate of transformation of Cy I to the other components. It is, however, possible that an enzymic mechanism also may be involved *in vivo*. Thus, mammalian tissues, *e.g.* skeletal muscle,[26,27] are known to be rich in enzymes which hydrolyze amide groups of the free amino acids L-glutamine and L-asparagine, and in the case of transglutaminase it has been shown that glutamine can serve as a substrate even when present in the peptide chain of a protein (see above). The validity of this alternative explanation for the formation of Cy II—Cy IV *in vivo* must, however, await further experimental evidence.

A large and rapidly increasing number of multiple molecular forms of enzymes and other proteins have been described during the last few years, and in many cases the different electrophoretic and chromatographic behaviour of the variants reflect a difference in the net charge of the protein molecules. There is a large body of evidence in support of the view that the type of heterogeneity demonstrated for cyt. c in the present study is fairly common, and a similar approach may therefore be useful to find out the physico-chemical basis for the heterogeneity of several other proteins.

Note added in proof. Recent studies on peptide maps of tryptic digests of subfractions Cy I and Cy II have revealed that Cy II gives two well resolved peptides (herein denoted peptide 1 and 2) in addition to the peptides found in Cy I. These two peptides both move towards the anode on electrophoresis in pyridine-acetate buffer at pH 6.5, peptide 2 faster than peptide 1. Amino acid analysis of acid hydrolysates revealed peptide 1 (yield approx. 30 %) to consist of five amino acids: aspartic acid, threonine, glutamic acid, alanine and lysine in the ratios 1.0:0.9:1.3:1.0:0.8, and peptide 2 (yield approx. 7 %) was found to be identical with peptide 1 except for a missing lysine. The composition of these two peptides fits only with the C-terminal tryptic peptides -Lys-Ala-Thr-Asn-GluCOOH (T-8-a) and -Ala-Thr-Asn-GluCOOH (T-2) as determined by Nakashima *et al.* (*J. Biol. Chem.* **241** (1966) 1166). Thus, the most labile amide in beef heart cytochrome c appears to be the asparagine in position 103 *i.e.* next to the C-terminal glutamic acid.

Acknowledgement. I wish to express my gratitude to Professor H. Theorell for his interest, helpful support and laboratory facilities, and to Docent Å. Åkeson for valuable discussions. Thanks are due to Mr. A. Baldesten for carrying out the amino acid analyses.

REFERENCES

1. Flatmark, T. *Acta Chem. Scand.* **18** (1964) 1656.
2. Flatmark, T. *Acta Chem. Scand.* **20** (1966) 1476.
3. Flatmark, T. *Acta Chem. Scand.* **18** (1964) 1517.
4. Åkeson, Å. and Theorell, H. *Arch. Biochem. Biophys.* **91** (1960) 319.

5. Spackman, D. H., Stein, W. H. and Moore, S. *Anal. Chem.* **30** (1958) 1190.
6. Conway, E. J. *Microdiffusion Analysis and Volumetric Error*, Crosby Lockwood & Son, London 1947.
7. Armstrong, J. McD., Coates, J. H. and Morton, R. K. In Falk, J. E., Lemberg, R. and Morton, R. K. (Eds.), *Haematin Enzymes*, Pergamon, Oxford 1961, p. 386.
8. Carlström, A. *Acta Chem. Scand.* **19** (1965) 2387.
8a. Archibald, R. M. *Chem. Rev.* **37** (1945) 161.
9. Vichery, H. B., Pucher, G. W., Clark, H. E., Chibnall, A. C. and Westall, R. G. *Biochem. J.* **29** (1935) 2710.
10. Gilbert, J. B., Price, V. E. and Greenstein, J. P. *J. Biol. Chem.* **180** (1949) 209.
11. Rosenblum, R. *Proc. Soc. Exptl. Biol. Med.* **119** (1965) 763.
12. Li, C. H., Geschwind, I. I., Dixon, J. S., Levy, A. L. and Harris, J. I. *J. Biol. Chem.* **213** (1955) 171.
13. Shepherd, R. G., Howard, K. S., Bell, P. H., Cacciola, A. R., Child, R. G., Davies, M. C., English, J. P., Finn, B. M., Meisenhelder, J. H., Moyer, A. W. and van der Scheer, J. *J. Am. Chem. Soc.* **78** (1956) 5051.
14. Lewis, U. J. and Cheever, E. V. *J. Biol. Chem.* **240** (1965) 247.
15. Harfenist, E. J. *J. Am. Chem. Soc.* **75** (1953) 5528.
16. Carpenter, F. H. and Chrambach, A. *J. Biol. Chem.* **237** (1962) 404.
17. Yasunobu, K. T., Nakashima, T., Higa, H., Matsubara, H. and Benson, A. *Biochim. Biophys. Acta* **78** (1963) 791.
18. Clarke, D. D., Mycek, M. J., Neidle, A. and Waelsch, H. *Arch. Biochem. Biophys.* **79** (1959) 338.
19. Neidle, A., Mycek, M. J., Clarke, D. D. and Waelsch, H. *Arch. Biochem. Biophys.* **77** (1958) 227.
20. Mycek, M. J., Clarke, D. D., Neidle, A. and Waelsch, H. *Arch. Biochem. Biophys.* **84** (1959) 528.
21. Mycek, M. J. and Waelsch, H. *J. Biol. Chem.* **235** (1960) 3513.
22. Folk, J. E. and Cole, P. W. *J. Biol. Chem.* **240** (1965) 2951.
23. Urry, D. W. *Proc. Natl. Acad. Sci. U.S.* **54** (1965) 640.
24. Flatmark, T. *Unpublished data.*
25. Flatmark, T. and Vesterberg, O. *Acta Chem. Scand.* **20** (1966) 1497.
26. Kazskhashnili, M. R. *Tr. Inst. Fiziol., Akad. Nauk. Gruz. SSR* **13** (1963) 209.
27. Silikova, G. I. and Polishckuk, S. M. *Ukr. Biokhim. Zh.* **36** (1964) 598.

Received February 25, 1966.