ENZYMATIC HYDROXYLATION OF PROLINE IN MICROSOMAL POLYPEPTIDE LEADING TO FORMATION OF COLLAGEN

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Communicated by C. B. Anfinsen, November 25, 1964

In a previous report,[1] we described a cell-free system derived from chick embryo which incorporated proline-C^{14} into protein and into collagen hydroxyproline. It was found that incorporation of proline-C^{14} into protein-bound proline began immediately and reached a maximum at about 1 hr. However, radioactivity did not appear in collagen hydroxyproline until after 30 min of incubation, when about 70 per cent of proline-C^{14} had already been incorporated into protein. This initial 30-min period was termed the lag phase, and further experiments using puromycin, ribonuclease, and anaerobic conditions led to the conclusions that hydroxylation of proline did not begin until after the lag phase and that proline already incorporated into a microsomal-bound polypeptide was the substrate for hydroxylation. These findings suggested that microsomes labeled with radioactive proline during the lag phase and then isolated could serve as substrate for proline hydroxylation. The present report describes the successful use of such prelabeled microsomes as a substrate and also some of the requirements and properties of chick embryo proline hydroxylase.

Materials and Methods.—Most of the materials and preparations of subcellular fractions have been described previously.[1] In the present experiments, the pH 5 precipitate was washed once with 0.02 *M* potassium acetate buffer, pH 5.0, and then dissolved with 0.02 *M* Tris·HCl buffer, pH 7.6, to give a final protein concentration of 8–10 mg/ml. A boiled supernatant fraction was prepared from the pH 5 supernatant by adjusting the pH to 7 with KOH and then heating at 100° for 5 min. After cooling to 0°, the denatured protein was removed by centrifugation.

Preparation of the ammonium sulfate fraction (A.S. fraction): To each ml of S-105 fraction, 0.63 gm ammonium sulfate was added gradually with stirring at 0° and the pH was maintained at 7 by the dropwise addition of KOH. Upon centrifugation of the suspension at 15,000 × *g* for 10 min, the precipitate floated on top. The entire tube was then stored at −15°. When the material was to be used, the liquid was drained from the tube and the precipitate suspended with a volume of 0.01 *M* imidazole·HCl buffer, pH 7.8, equal to about one half the original S-105 volume. The suspension was then dialyzed in size 27/32 tubing for 2 hr against 2 changes of 0.01 *M* imidazole buffer. During dialysis, the precipitate went into solution completely and the final protein concentration was usually about 20 mg/ml.

Preparation of microsomes prelabeled with radioactive proline: Incubations were carried out in 50-ml Erlenmeyer flasks designed for evacuation, and contained the following components: pH 7.6 Tris·HCl, 500 μmoles; ATP, 20 μmoles; creatine phosphate, 200 μmoles; $MgCl_2$, 40 μmoles; KCl, 200 μmoles; sucrose, 2.5 mmoles; microsomal protein, 50–60 mg; pH 5 enzyme protein, 20–30 mg; and either 5 μc proline-C^{14} or 25 μc 3,4-H^3-proline. The final volume was 10 ml. The flasks were kept at 0° until they had been evacuated and flushed with N_2 four times. The stopcocks were closed with N_2 in the flasks, and incubation was carried out, in most cases, for 25 min at 37° without shaking. The flasks were then quickly chilled to 0°, the contents transferred to tubes and centrifuged at 105,000 × *g* for 75–90 min. The pellets were rinsed with cold 0.25 *M* sucrose, the tubes drained, wiped, and then stored at −15°. Microsomes labeled with proline-H^3 are denoted as H^3-microsomes, while those labeled with proline-C^{14} are denoted as C^{14}-microsomes. When the microsomes were to be used, they were thawed gradually by placing 1 ml of cold 0.25 *M* sucrose over the pellet and keeping the tube at 0° for about 1 hr. More 0.25 *M* sucrose

was then added, and the pellet was gently resuspended to give a final protein concentration of approximately 10 mg/ml.

Procedure for measuring hydroxylation of prelabeled microsomes: Hydroxylation experiments were carried out using the following procedure, except where indicated in the figure legends. Incubations were carried out at 37°, with shaking, in 25-ml stoppered Erlenmeyer flasks. The following components were present in a final volume of 5 ml: imidazole·HCl buffer, pH 7.8, 250 μmoles; sucrose, 1.25 mmoles; prelabeled microsomal protein, 7–16 mg; A.S. fraction protein, 30–46 mg; boiled supernatant, 1.0 ml. After 25 min of incubation the flasks were chilled to 0° and the contents transferred to centrifuge tubes. Each flask was rinsed with 1 ml of water which was added to the reaction mixture, and 0.6 ml of 50% trichloroacetic acid (TCA) was then added with stirring. Hot TCA extracts were prepared from the precipitates and were hydrolyzed and treated as described previously.[1] Radioactivity in proline and hydroxyproline was then measured.[2] Results are expressed as total cpm of protein-bound proline and hydroxyproline after correction for recovery in the assay. In some instances the results are also given as the per cent of the protein-bound proline hydroxylated, in order to compare the present results with those found *in vivo* and with the previously reported system. Protein was measured by the method of Warburg and Christian.[3] Ascorbic acid was measured by the method of Roe[4] modified so that heating was carried out at 50° for 30 min rather than at 37° for 4 hr. Assays were carried out with and without the addition of $CuSO_4$ in order to measure ascorbic acid specifically.

Results.—Initial experiments were carried out to determine whether hydroxylation of proline could be observed after radioactive proline had been incorporated into microsomal polypeptide during the lag phase. For this purpose, dialyzed S-15 fraction was incubated for 30 min with proline-C^{14} and an ATP-generating system as described previously.[1] Incubations were carried out under nitrogen to ensure minimal hydroxylation. The microsomes were then separated and immediately resuspended in various media as described in detail under Table 1. The resuspended microsomes were then reincubated aerobically for another 60 min and assayed for protein-bound proline-C^{14} and hydroxyproline-C^{14}. It may be seen (Table 1) that isolated microsomes which were not subjected to the second incubation contained only a small amount of labeled hydroxyproline. This level was not increased significantly by incubation with a boiled supernatant fraction. The amount of labeled hydroxyproline was increased, however, by the addition of either the original S-105 fraction, still containing proline-C^{14} and the ATP-generating system, or to a lesser extent by fresh S-105 fraction containing no added cofactors. The fraction of microsomal proline which was hydroxylated was similar in both cases. This experiment indicated that microsomes prelabeled with radioactive proline could be used as a substrate for proline hydroxylase.

In order to obtain more highly labeled microsomes, preparations were made using a system composed of microsomes plus pH 5 enzyme and the ATP-generating sys-

TABLE 1

USE OF PRELABELED MICROSOMES AS SUBSTRATE FOR PROLINE HYDROXYLATION

Additions to prelabeled microsomes	Radioactivity in Protein-Bound Imino Acids, cpm		Per cent hydroxylation
	Proline	Hydroxyproline	
None	7,720	117	1.5
Boiled supernatant	7,420	144	1.9
Fresh S-105	6,730	331	4.9
C^{14}-S-105	11,030	580	5.3

In order to label the microsomes, dialyzed S-15 fraction containing 86 mg protein was incubated for 30 min under N_2 as described in the text. The microsomes were then isolated by centrifugation at 105,000 × *g*. The pellets were rinsed with 0.25 *M* sucrose and immediately resuspended in 0.25 *M* sucrose containing the additions indicated above. The final volume was 10 ml. The suspensions were reincubated aerobically for 60 min at 37° with shaking. The reaction was stopped by adding 1.1 ml of 50% TCA and the precipitates were treated as described under *Methods*. The per cent hydroxylation = [(hydroxyproline, cpm)/(proline, cpm)] × 100.

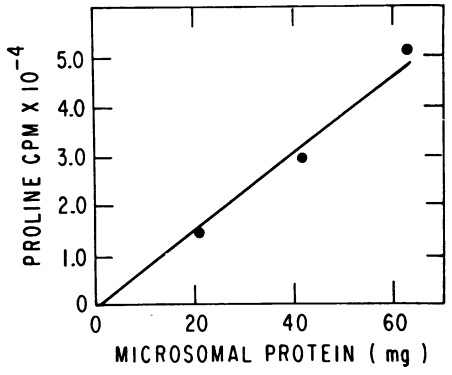

FIG. 1.—The incorporation of proline-C^{14} into hot TCA-extractable, microsomal protein under anaerobic conditions. Incubations were carried out as described under *Methods*, paragraph 3. Each flask contained 33 mg of pH 5 enzyme protein. The amount of labeled hydroxyproline found averaged 0.80% of the proline radioactivity.

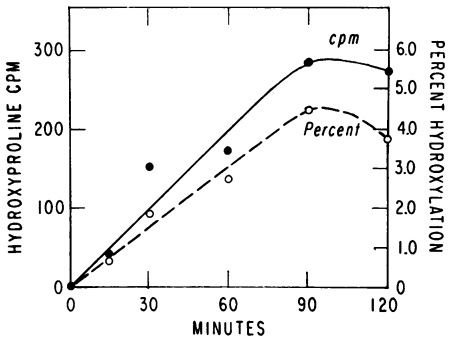

FIG. 2.—Time-course of hydroxylation of prelabeled microsomes. Incubations were carried out as described under *Methods*, except that separate flasks were used for each time point. The concentration of prelabeled C^{14}-microsomal protein was 2.0 mg/ml and of A.S. fraction protein, 7.8 mg/ml. Per cent hydroxylation was calculated as described under Table 1.

tem. With this incorporation system, most of the free proline pool was eliminated, so that the specific activity of the added radioactive proline was not diluted to a great extent by endogenous proline. Figure 1 shows the amount of incorporation of proline-C^{14} into hot TCA-extractable, protein-bound proline of microsomes. The incorporations shown were obtained with a constant amount of pH 5 enzyme and varying amounts of microsomal protein. It may be seen that incorporation was linear at least up to 63 mg of microsomal protein. Anaerobically labeled microsomes were usually prepared by adding the maximum amount of microsomes possible in a single flask. It was found that after microsomes had been labeled and isolated by centrifugation, they could be stored as a pellet in the Deepfreeze for as long as 7 weeks and still be used to obtain efficient hydroxylation.

Properties of the proline hydroxylating system using prelabeled microsomes as substrate: As shown in Table 2, it was found that the S-105 fraction could be replaced

TABLE 2

DEPENDENCE ON A SOLUBLE PROTEIN FRACTION AND A BOILED SUPERNATANT FRACTION

Expt.	Additions to prelabeled microsomes	Radioactivity in Protein-Bound Imino Acids, cpm: Proline	Hydroxyproline	Per cent hydroxylation
1	None	13,070	77	0.59
	Boiled supernatant	11,920	109	0.92
	A.S. fraction	7,460	81	1.1
	A.S. fraction plus boiled supernatant	7,600	478	6.3
	S-105	8,500	234	2.8
2	None	3,770	27	0.59
	Boiled supernatant	3,520	107	3.0
	A.S. fraction	2,330	91	3.9
	Boiled supernatant + boiled A.S. fraction	3,310	271	8.2
	Boiled supernatant + A.S. fraction	2,690	244	9.1

Microsomes were prelabeled with radioactive proline as described under *Methods*. The C^{14}-microsomes used in expt. 1 had been stored for 1 week and in expt. 2 for 7 weeks prior to use. Incubations to obtain hydroxylation were carried out using the conditions described under *Methods* except that boiled supernatant fraction or A.S. fraction was added as indicated above. The concentrations of prelabeled microsomal protein used were: expt. 1, 1.6 mg/ml; expt. 2, 1.2 mg/ml. The concentrations of A.S. fraction protein were: expt. 1 6.4 mg/ml; expt. 2, 8.5 mg/ml. In expt. 1, 6.5 mg/ml S-105 protein were used. The boiled A.S. fraction was prepared by heating the appropriate amount in the flask to be used for incubation at 100° for 5 min. After cooling the flask to 0°, the remaining components were added.

by a boiled supernatant fraction plus a soluble protein fraction (A.S. fraction). Neither fraction alone gave full activity but both together showed more activity than the S-105 fraction alone. Furthermore, activity was maximal even if the A.S. fraction were heated. This suggested that two nonenzymatic factors were involved in the reaction and also that the enzyme was located in the microsomal fraction.

Figure 2 shows that the time course of hydroxylation of prelabeled C^{14}-microsomes, plotting either total radioactivity or per cent hydroxylation, was linear for about 90 min, after which time it fell off. The decrease in hydroxylated product after 90 min is probably due to proteolytic activity or loss of microsomal-bound polypeptide. Some loss of protein-bound proline radioactivity was always noted during incubations, especially when the A.S. fraction was present. The present curve (Fig. 2) would correspond to the portion of the curve after the lag phase in the time course previously observed.[1] This is further evidence that the substrate for hydroxylation is synthesized during the lag phase and thereafter hydroxylation occurs at a linear rate.

The effect of puromycin: In our previous studies, it was observed that when puromycin was added at the beginning of the incubation period, incorporation of proline-C^{14} into both protein-bound proline and collagen hydroxyproline was almost completely inhibited. When puromycin was added at the end of the lag phase, however, there was practically no inhibition of the incorporation of proline-C^{14} into collagen hydroxyproline, although labeling of collagen hydroxyproline did not begin until that time. The additional observations that anaerobiosis during the lag phase did not affect incorporation into collagen hydroxyproline while anaerobiosis after the lag phase or during the entire incubation period was inhibitory led to the conclusion that hydroxylation began after the lag phase. Those experiments on the effects of puromycin and anaerobiosis strongly suggested that proline had already been incorporated into peptide-linkage before it was hydroxylated.

The effect of puromycin was confirmed using the present system. The data in

TABLE 3

EFFECT OF PUROMYCIN ON HYDROXYLATION OF PROLINE IN PRELABELED MICROSOMES

Expt.	Conditions	Hydroxyproline, cpm
1	Complete system	294
	Plus puromycin, 0.2 μmole/ml	389
	Minus A.S. fraction	88
2	Complete system	244
	Plus puromycin, 0.2 μmole/ml	290
	Minus A.S. fraction	107

Incubations to obtain hydroxylation were carried out as described under *Methods* except that puromycin was added as indicated. The concentrations of C^{14}-microsomal protein were: expt. 1, 2.0 mg/ml; expt. 2, 1.4 mg/ml. The concentrations of A.S. fraction protein were: expt. 1, 7.8 mg/ml; expt. 2, 8.6 mg/ml. The results without A.S. fraction are given to indicate the extent of stimulation of hydroxylation by the complete system.

TABLE 4

REPLACEMENT OF BOILED SUPERNATANT FRACTION BY ASCORBATE

Addition to prelabeled microsomes	Radioactivity in Protein-Bound Imino Acids, cpm: Proline	Hydroxyproline	Per cent hydroxylation
None	9020	53	0.59
Ascorbate	8120	73	0.90
Boiled C^{14}-microsomes plus ascorbate plus A.S. fraction	5190	52	1.0
Ascorbate plus boiled A.S. fraction	6580	540	8.2
Ascorbate plus A.S. fraction	5470	523	9.6

Incubations were carried out as described under Table 2. The concentration of microsomal protein was 3.2 mg/ml and A.S. fraction protein 9.2 mg/ml. C^{14}-microsomes had been stored for 1 week prior to use. The concentration of ascorbate used was 1.0 μmole/ml. Where boiled C^{14}-microsomes are indicated, these were used in place of the normal, prelabeled microsomes.

Table 3 indicate that there was no inhibition of the hydroxylation of prelabeled C^{14}-microsomes. If any effect was noted at all, it was a stimulation of hydroxylation. It should be pointed out that the concentration of puromycin used in these experiments, 0.20 μmole/ml, inhibited protein synthesis in the chick embryo S-15 system to the extent of 99 per cent.

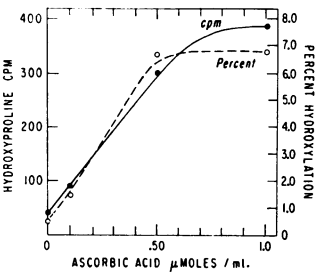

FIG. 3.—Effect of varying ascorbate concentration on hydroxylation of prelabeled microsomes. Incubations were carried out as described under *Methods*, except that varying amounts of sodium ascorbate were used in place of the boiled supernatant fraction. The concentrations of prelabeled microsomal protein and A.S. fraction protein were as indicated under Fig. 2.

The effect of various hydrogen donors on hydroxylation: It was found that ascorbic acid completely replaced the boiled supernatant fraction, as shown in Table 4. However, the A.S. fraction was still required, either boiled or unboiled. In this experiment it was also found that boiling the microsomes resulted in a complete loss of activity, providing further evidence for localization of the hydroxylating enzyme in the microsomes. It might be argued that boiling the microsomes disrupted the structure of the microsomes. However, evidence against this argument is the finding that as much protein-bound proline radioactivity was observed in the hot TCA extract from the boiled microsomes as in that from unboiled microsomes under comparable conditions. The saturating concentration of ascorbate was found to be 1.0 μmole/ml as shown in Figure 3.

Some other hydrogen donors were able to replace the boiled supernatant requirement and the relative activities of these compounds are shown in Table 5. Although ascorbic acid at 1.0 μmole/ml was most effective, 2-amino-4-hydroxytetrahydrodimethylpteridine ($DMPH_4$), a cofactor for phenylalanine[5] and tyrosine[6] hydroxylases, was almost as effective at a much lower concentration, 0.20 μmole/ml. All of the compounds were tested in the presence of the A.S fraction.

It was thought that the A.S. fraction might contain a bound pteridine which was reduced by ascorbic acid. Protein-bound folic acid derivatives which can be maintained in a reduced state by ascorbate have been observed in red blood cells.[7] As may be seen in Table 6, however, ascorbate and dimethylpteridine together did not give full activity when compared to either ascorbate plus the A.S. fraction or reduced pteridine plus the A.S. fraction. In this experiment, reduced pteridine at 0.20 μmole/ml was not quite as active as ascorbate at 1.0 μmole/ml. It was also thought that the A.S fraction might be acting as a nonspecific protective agent for the active hydrogen donor, but when mercaptoethanol was tested as a replacement for the A.S. fraction, it had no effect.

Discussion.—The observation that microsomes can be labeled with radioactive proline during a short, anaerobic incubation and then used as substrate for proline hydroxylation strongly supports our previous conclusions regarding the sequence of events leading to the appearance of radioactive hydroxyproline in collagen.[1] Furthermore, puromycin had no inhibitory effect on the appearance of labeled collagen hydroxyproline in prelabeled microsomes. This is additional evidence that the radioactive proline bound to the microsomes must be in peptide linkage, since current evidence indicates that puromycin inhibits protein synthesis by dis-

TABLE 5

RELATIVE ACTIVITY OF HYDROGEN DONORS FOR REPLACING THE BOILED SUPERNATANT FRACTION

Hydrogen donor	Concentration, μmoles/ml	Radioactivity in hydroxyproline, cpm	Relative activity*
Ascorbate	1.0	263	100
$DMPH_4$	0.20	250	93
TPNH	1.0	169	52
EtSH	1.0	130	33
GSH	1.0	73	3.5
DPNH	1.0	69	1.5
None		66	0

* The relative activity was calculated by assuming minimal activity to be that when no additions were made, other than the A.S. fraction, maximal activity, that when ascorbate and A.S. fraction were added.

Incubations to obtain hydroxylation were carried out as described under *Methods* except that the hydrogen donors indicated above were used to replace the boiled supernatant fraction. The flask containing dimethylpteridine was covered with aluminum foil to prevent light from entering. The concentration of prelabeled microsomal protein was 2.6 mg/ml and of A.S. fraction protein, 7.4 mg/ml. C^{14}-microsomes were used and had been stored for 1 week prior to use.

TABLE 6

EFFECT OF ASCORBATE AND DIMETHYLPTERIDINE ON PROLINE HYDROXYLATION

Additions to prelabeled microsomes	Radioactive hydroxyproline, cpm	Per cent hydroxylation
None	53	0.59
$DMPH_4$	56	0.76
Ascorbate	73	0.90
$DMPH_4$ + Ascorbate	104	1.9
$DMPH_4$ + EtSH	53	0.67
$DMPH_4$ + A.S. fraction	388	7.8
$DMPH_4$ + A.S. fraction + ascorbate	478	9.0
$DMPH_4$ + A.S. fraction + EtSH	405	7.7
Ascorbate + A.S. fraction	523	9.6

Incubations were carried out as described under Table 5 except that light was excluded. The concentration of microsomal protein was 3.2 mg/ml and of A.S. fraction protein, 9.2 mg/ml. The C^{14}-microsomes which were used had been stored for 7 weeks prior to use. The following concentrations of hydrogen donors were used: ascorbate, 1.0 μmole/ml; $DMPH_4$, 0.20 μmole/ml; EtSH, 1.0 μmole/ml.

placing newly formed peptide chains from ribosomal-bound sRNA.[8, 9]

Recently, several articles have appeared which report the finding of sRNA-hydroxyproline in the soluble fraction of chick embryo and carageenan granuloma systems.[10–12] The significance of these findings remains to be established, since in no case was the reported sRNA-hydroxyproline proved to be an intermediate in the biosynthesis of collagen hydroxyproline.[13] On the contrary, we have shown that using prelabeled microsomes, there is no further requirement for a radioactive proline intermediate from the soluble fraction. The labeled microsomal-bound polypeptide can be converted to microsomal-bound collagen by a system composed of a hydrogen donor plus another nonenzymatic factor in the soluble protein faction. In addition, evidence has been obtained which establishes the similarity between the unhydroxylated, microsomal-bound polypeptide and the microsomal-bound collagen, in that both are degraded by a highly specific preparation of bacterial collagenase.[14]

Ascorbic acid was first reported to be involved in collagen synthesis by Robertson and Schwartz[15] who found that carageenan granulomas in scorbutic guinea pigs contained much less collagen than those in normal guinea pigs. Since ascorbic acid has been shown to act as a hydrogen donor in several hydroxylase reactions, including dopamine-β-hydroxylase[16] and phenolase,[17] it has been thought that proline hydroxylation might be the step in collagen synthesis affected by ascorbic acid deprivation. Stone and Meister[18] and Robertson and Hewitt[19] have shown that tissue minces or breis prepared from granulomas of scorbutic guinea pigs exhibit a reduced incorporation of proline into collagen hydroxyproline as compared to preparations from normal guinea pigs. The addition of ascorbic acid to the preparations *in vitro* brought the incorporation back to normal levels. These findings, although indirect, suggested that the hydroxylation of proline was affected.

Our experiments indicate that one of the requirements for the hydroxylation of protein-bound proline, the boiled supernatant fraction, can be completely replaced

by ascorbic acid. The saturating concentration of ascorbate, 1.0 μmole/ml, is comparable if not lower than concentrations required for other hydroxylase enzymes. The requirement for ascorbate is not specific, since tetrahydrodimethylpteridine is also an effective replacement for the boiled supernatant fraction. Which, if either, of these hydrogen donors acts physiologically remains to be determined. The boiled supernatant fraction was found to contain amounts of ascorbic acid which resulted in concentrations of 0.06–0.11 μmole/ml in the final incubation mixture. There was no trace of any reduced pteridine capable of acting as a cofactor in a bacterial phenylalanine hydroxylase system which can utilize tetrahydrodimethylpteridine.[20] There also is a possibility that ascorbic acid is functioning indirectly to produce reduced pyridine nucleotide since TPNH showed some activity for replacing the boiled supernatant. There was also found to be a requirement for a nonenzymatic factor in the soluble protein fraction. This factor could be a protein-bound metal, since ferrous ion, in addition to a hydrogen donor, has been shown to be a requirement for several hydroxylases.

The extent of hydroxylation obtained with the present system is comparable to that obtained in the cruder system.[1] In the crude system, the amount of labeled collagen hydroxyproline found was about 10 per cent of the amount of radioactive protein-bound proline extracted from microsomes by hot TCA. When intact chick embryos are injected with proline-C^{14}, the amount of labeled collagen hydroxyproline present is also about 10 per cent of the labeled protein-bound proline extracted with hot TCA from the microsomes. This value of 10 per cent may represent a steady-state concentration of microsomal collagen which cannot be exceeded. The rate of release of completed collagen chains from the microsomes may determine the level of collagen in tissues. As a consequence, the rate of hydroxylation of the proline-rich microsomal-bound polypeptide and the concentrations of hydroxylase cofactors would play a vital role in controlling collagen synthesis. Such a situation would explain the relationship between ascorbic acid and collagen synthesis found in many biological systems, if in fact ascorbic acid is the specific cofactor of proline hydroxylase.

Summary.—It has been found that the conversion of unhydroxylated microsomal-bound polypeptide to collagen requires a hydrogen donor, another nonenzymatic factor in the soluble protein fraction and microsomes. Ascorbate or 2-amino-4-hydroxydimethylpteridine can serve as the hydrogen donor. Proline hydroxylase appears to be located in the microsomal fraction.

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[1] Peterkofsky, B., and S. Udenfriend, *J. Biol. Chem.*, **238**, 3966 (1963).

[2] Peterkofsky, B., and D. J. Prockop, *Anal. Biochem.*, **4**, 400 (1962).

[3] Warburg, O., and W. Christian, *Biochem. Z.*, **310**, 384 (1941).

[4] Roe, J. H., in *Methods of Biochemical Analysis*, ed. D. Glick (New York: Interscience Publishers, Inc., 1954), vol. 1, p. 137.

[5] Kaufman, S., *J. Biol. Chem.*, **234**, 2677 (1959).

[6] Nagatsu, T., M. Levitt, and S. Udenfriend, *J. Biol. Chem.*, **239**, 2910 (1964).

[7] Iwai, K., P. M. Luttner, and G. Toennies, *J. Biol. Chem.*, **239**, 2365 (1964).

[8] Arlinghaus, R., G. Favelukes, and R. Schweet, *Biochem. Biophys. Res. Commun.*, **11**, 92 (1963).

[9] Nathans, D., these PROCEEDINGS, **51**, 585 (1964).

[10] Manner, G., and B. S. Gould, *Biochim. Biophys. Acta*, **72**, 243 (1963).

[11] Coronado, A., E. Mardones, and J. E. Allende, *Biochem. Biophys. Res. Commun.*, **13**, 75 (1963).

[12] Jackson, D. S., D. Watkins, and A. Winkler, *Biochim. Biophys. Acta*, **87**, 152 (1964).

[13] All attempts in this laboratory to demonstrate the formation of sRNA-hydroxyproline from proline have been unsuccessful (B. Peterkofsky, in Ph.D. dissertation, George Washington University, Washington, D.C., Feb. 1964, p. 72, and also unpublished observations of B. Peterkofsky and of A. Gottlieb).

[14] Gottlieb, A., B. Peterkofsky, and S. Udenfriend, in preparation.

[15] Robertson, W. van B., and B. Schwartz, *J. Biol. Chem.*, **201**, 689 (1953).

[16] Levin, E. Y., B. Levenberg, and S. Kaufman, *J. Biol. Chem.*, **235**, 2080 (1960).

[17] Mason, H. S., W. L. Fowlks, and E. Peterson, *J. Am. Chem. Soc.*, **77**, 2914 (1955).

[18] Stone, N., and A. Meister, *Nature*, **194**, 555 (1962).

[19] Robertson, W. van B., and J. Hewitt, *Biochim. Biophys. Acta*, **49**, 404 (1961).

[20] This assay was kindly performed by Dr. G. Guroff, using phenylalanine hydroxylase obtained from a phenylalanine-induced Pseudomonas strain.

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*DETERMINATION OF STOICHIOMETRY AND EQUILIBRIUM CONSTANTS FOR REVERSIBLY ASSOCIATING SYSTEMS BY MOLECULAR SIEVE CHROMATOGRAPHY**

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Communicated by Albert L. Lehninger, November 23, 1964

The supratertiary architecture and self-assembly characteristics of enzyme complexes, viruses, membranes, immunochemical, and mechanochemical systems are central problems in molecular biology.[1, 2] Among the simplest prototypes of such systems are association-dissociation phenomena of proteins and protein subunits. Recent investigations demonstrate that in a number of systems these phenomena occur only at very low concentrations.[3, 4] The molecular sieve technique has special advantages for the study of such systems in the lower ranges of concentration for which only spectrophotometric or biological activity assay is available. Furthermore, elution behavior can often be related in a straightforward way to intrinsic molecular properties such as the diffusion coefficients,[7] sedimentation coefficients,[9, 10] and molecular weights.[11–13] It is the purpose of this paper to show that this technique may be used for quantitative analysis of the number of components and their equilibria in such dilute systems.

Recently, Winzor and Scheraga[5, 6] demonstrated that molecular sieve chromatography possesses all of the qualitative features predicted by Gilbert[15] for the sedimentation of a rapidly reversible, polymerizing system. However, no quantitative theory of the molecular sieve applied to such systems has been reported to date, although the Gilbert theory emphasized the analogy of sedimentation and chromatography. In this paper a quantitative theory for the molecular sieve is developed, and the formal relationship between the two transport methods is established. The theoretical predictions are tested against experimental data for the monomer-hexamer interaction of α-chymotrypsin and for the reversible dissociation of isoelectric human carboxyhemoglobin.