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The Biosynthesis of Pulvilloric Acid. II. Studies on Incorporation of Radioactive Precursors*

S. W. Tanenbaum and S. Nakajima

ABSTRACT: The biosynthesis of pulvilloric acid, an unstable secondary metabolite produced by *Penicillium pulvillorum*, was studied with the use of ^{14}C-labeled small precursor molecules. Radioactive pulvilloric acid obtained from acetate-1-^{14}C supplementation was subjected to a complete series of degradations. Of the fifteen carbon atoms in the molecule, nine were assayed individually for their specific radioactivities.

The results revealed that the acetate–polymalonate pattern, though possibly not involving a single C_{14} polyketide chain, was operative in its biogenesis. Following growth experiments in the presence of $H^{14}COOH$ and partial degradation of the resultant antibiotic, the carboxyl function of pulvilloric acid was demonstrated to have arisen from the C_1-transfer pool. These findings parallel the known pathway of biosynthesis of citrinin, a metabolite closely related in structure. Ethyl hexanoate-1-^{14}C was fed to the growing mold to test the possible role of hexanoyl coenzyme A or other C_6-4′-phosphopantetheine intermediate as a "starter" in pulvilloric acid formation. However, the antibiotic which was isolated in this experiment exhibited an isotopic distribution pattern which reflected its prior degradation to the level of acetate-1-^{14}C. Alternative pathways for the biosynthesis of pulvilloric acid, and their biogenetic relationships to structurally similar polyacetate metabolites and to the formation of fatty acids, are discussed.

Pulvilloric acid (I) is a metabolite accumulated by *Penicillium pulvillorum* (Brian *et al.*, 1957) which is closely related in its structure to ascochitine (Ia; Iwai and Mishima, 1965) and to citrinin (II), also secondary metabolites of higher fungi. Of biosynthetic interest is the fact that pulvilloric acid contains a *n*-pentyl side chain at a carbonyl-derived point of juncture with its benzopyran ring system; it thus presents biogenetic features which may also relate it to many lichen depsides and depsidones (Asahina and Shibata, 1954) which embody the olivetolcarboxylic acid fragment (III).

In light of the knowledge that the biosynthesis of citrinin (Birch *et al.*, 1958; Schwenck *et al.*, 1958; Rodig *et al.*, 1966) has been shown to involve the acetate–polymalonate condensation route, followed by donation of extracyclic methyl groups as well as the carboxyl function from C_1-transfer systems, a grossly similar pathway for pulvilloric acid formation was anticipated. From the work of Curtis *et al.* (1968) with mutant strains of *Penicillium citrinum* and their accumulation products, it would appear furthermore that *C*-acyl-*o*-orsellinic acids might be involved in the biosynthesis

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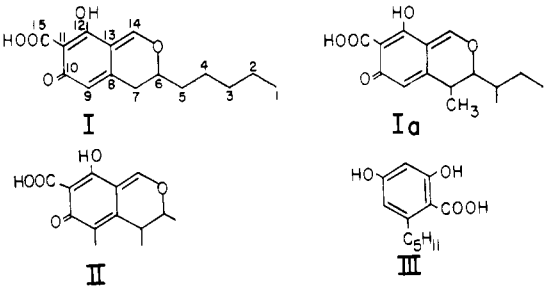

of Ia, Ib, or II. For pulvilloric acid biogenesis, such hypothetical intermediates could be formed after chain extension of a fatty acid fragment as large as β-ketooctanoate with malonyl units; or else after selective reductions at the initiating C_5 portion of the extended, template-bound C_{14} polyketide.

This paper reports results of several isotope incorporation experiments in relation to the above considerations. Supplementation of growing cultures of *P. pulvillorum* with acetate-1-^{14}C followed by a complete degradation of the resultant pulvilloric acid showed the essential polyacetate nature of this antibiotic. As with citrinin, the labeling in pulvilloric acid after formate-^{14}C incorporation indicated the origins of its exocyclic carboxyl in the single-carbon transfer pool. An attempt to examine the participation of a fatty acyl-4′-phosphopantetheine "starter" in pulvilloric acid formation by feeding ethyl hexanoate-1-^{14}C to the mold, led however to results which demonstrated only prior breakdown of the hexanoate to the level of acetate.

Experimental Procedure

Growth of Cultures and Isolation of Radioactive Pulvilloric Acid. An improved strain of *P. pulvillorum* ACC 1124 (Nakajima and Tanenbaum, 1968) was used. General growth conditions and microscale isolation procedures for pulvilloric acid are described in part I of this series (Tanenbaum and Nakajima, 1969). For radioisotope incorporation studies, cultures were grown in 2.8-l. fernbach flasks, containing 500 ml of medium. After four days at 26° on 5% glycerol Czapek-Dox medium with corn steep liquor, sodium formate-^{14}C, and sodium acetate-1-^{14}C were each added, by use of a 6-in. syringe needle attached to a luer-lock glass syringe. By this means, their aqueous solutions were expressed underneath the mycelial mats. Ethyl hexanoate-1-^{14}C was added in the same way, but was first dissolved in 1.0 ml 95% ethanol. Growth was allowed to proceed in stationary cultures for an additional 11 days. Yields of pure, radioactive pulvilloric acid ranged from 200 to 400 mg per flask.

Degradative Methods. Details of the stepwise degradations which were employed to ascertain the patterns of isotope distributions in pulvilloric acid are given in part I (Tanenbaum and Nakajima, 1969). In the case of acetate-1-^{14}C supplementation, the isolated pulvilloric acid was diluted 2.5-fold with unlabeled material, later followed by an additional 20-fold dilution with 1-(3,5-dimethoxyphenyl)-2-heptanol. After growth in the presence of ethyl hexanoate-1-^{14}C, the pulvilloric acid was only initially diluted with a 2.5-fold quantity of carrier. No dilutions were required for partial degradation of the pulvilloric acid which was obtained after growth of *P. pulvillorum* in the presence of formate-^{14}C.

Synthesis of Carrier Pulvilloric Acid. Since pulvilloric acid has a poor shelf life, it was not always possible to have sufficient, pure material at hand for carrier purposes. This problem was resolved by transformation of accumulated stocks of pulvilloric acid, by way of alkaline rearrangement, to the more stable 1-(3,5-dihydroxyphenyl)-2-heptanol. This was reconverted, as needed, into pulvilloric acid by carboxylation and then formylation and cyclization, using the methods described by Bullimore *et al.*, 1967) for the synthesis of the (±)-antibiotic. The optically active intermediate 1-(3,5-dihydroxy-4-carboxyphenyl)-2-heptanol, melted at 128°. Synthetic pulvilloric acid made by this procedure was identical in all respects with the authentic natural product.

Radioactivity Determinations. Radioactivity measurements were carried out using a Packard Tri-Carb Model 2002 liquid scintillation counter. Depending upon their solubilities, ^{14}C-labeled intermediates were counted in dioxane scintillator (7.0 g of 2,5-diphenyloxazole, 300 ml of 1,4-bis[2-(4-methyl-5-phenyloxazole)], and 100 g of naphthalene per l. of solvent), or in toluene scintillator (5.0 g of 2,5-diphenyloxazole and 300 ml of 1,4-bis[2-(4-methyl-5-phenyloxazole)] per l. of solvent). In many cases, values given in the tables average direct counting data so obtained, taken together with radioactivity determinations found after combustion of the samples into $BaCO_3$. Conversion and counting of samples as $BaCO_3$ was carried out by further modification of the method of Woeller (1961). The apparatus consisted of a side-arm tip-in Van Slyke–Folch adapter (*cf.* Calvin *et al.*, 1949), which was connected by a 24/40 joint to the modified receptacle (Weyman *et al.*, 1967) which accommodated a standard counting vial. The vial was charged with 5.0 ml of a mixture of β-phenethylamine (Packard)–1-propanol–dioxane 2:1:2). The apparatus was evacuated with a water aspirator, and substrates were oxidized with Van Slyke's mixture. Samples of $BaCO_3$ which were trapped for counting purposes in the above solvent mixture, were liberated with 70% perchloric acid. After standing for 5 hr, the apparatus was opened to the atmosphere, 15 ml of dioxane scintillator fluid was added to the trapping mixture, and the vial was capped and then counted. In this fashion, essentially quantitative recoveries with counting efficiencies of 70–90% were achieved. All samples counted were suitably corrected for quenching, efficiency, and background.

Radioactive Precursors. Sodium acetate-1-^{14}C and sodium formate-^{14}C were purchased from New England Nuclear Corp. Sodium hexanoate-1-^{14}C was quantitatively converted into its ethyl ester by treatment of the liberated fatty acid in ether with an excess of diazoethane.

Results

When *P. pulvillorum* was grown on modified glycerol Czapek-Dox medium in the presence of sodium formate-^{14}C, 2.4% of the initial label was found in the resultant pulvilloric acid. An equal amount of radioactivity remained in the ethereal extract following pulvilloric acid removal, while 4.7% could not be extracted from the acidified fermentation beer. The washed, dried mycelium incorporated 23.7% of the total starting radioactivity. No attempt to obtain a radiochemical balance was made in this or in subsequent experiments since preliminary investigations showed that a marked diminution in product yields accompanied the collection

TABLE I: Distribution of Radioactivity in Pulvilloric Acid from Radiolabeled Formate.[a]

Compd or Fragment Counted	Sp Act. (dpm/mmole)	% Act.
Pulvilloric acid	5.14×10^6	100
1-(3,5-Dihydroxyphenyl)-2-heptanol	3.85×10^5	7.5
$BaCO_3$ (C_{14})	0	0
$BaCO_3$ (C_{15})	3.71×10^6	72

[a] *P. pulvillorum* ACC 1124 was grown on the modified Czapek-Dox medium. After 4 days, 2.41 mg of $H^{14}COONa$ (0.106 mCi) was added. Subsequent operations are described under Experimental Section.

procedures (Tanenbaum and Bassett, 1959) used in this Laboratory for obtaining respiratory carbon dioxide. The distribution of radioactivity in the purified pulvilloric acid obtained in this experiment is given in Table I. Partial degradation of the antibiotic demonstrated that over 70% of the total activity (0.72 C) was present in the exocyclic carboxyl group (C_{15}). No activity was detected in C_{14} (a carbon biogenetically derived from an acetate carboxyl), while a relatively small amount of the tracer (7.5%) was distributed in some undetermined manner, among the remaining carbons of the 1-(3,5-dihydroxyphenyl)-2-heptanol. The results however, are in essential agreement with earlier studies (Birch *et al.*, 1958; Schwenck *et al.*, 1958) on citrinin biosynthesis, in which it was shown that its carboxyl group analogously originates in the C_1-transfer pool. Failure to account for a radiochemical balance cannot be ascribed in any great degree to extensive degradation of the rest of the molecule during alkaline hydrolysis, since scrambling of radioactivity into this carbon was not noted in the acetate-1-^{14}C or ethyl hexanoate-1-^{14}C experiments (see below).

Growth of *P. pulvillorum* in the presence of acetate-1-^{14}C led to incorporation of 1.6% of the total initial radioactivity into the principal secondary metabolite. Approximately 0.8% of the label remained in the ether extract of the beer after pulvilloric acid isolation, while 7.8% of the activity was not solvent soluble. The washed, dried mycelium contained 43.9% of the starting radioactivity. The complete degradation of this sample of pulvilloric acid was carried out by the series of reactions delineated in Figure 2 of part I (Tanenbaum and Nakajima, 1969). The determination of radioactivity of 12 of the 15 carbon atoms of acetate-labeled pulvilloric acid is given in Table II. The values for the other three carbons are assigned by difference. Of those carbons whose specific activities were individually ascertained, C_2, C_4, and C_6 averaged 0.94 C (theoretical 1.00 C, on the assumption of incorporation of 7 acetate units into the molecule). The specific activity of C_{14} (0.86 C) obtained by alkaline rearrangement of pulvilloric acid, is slightly lower than can be explained on the basis of counting error. The other product of this reaction, C_{15}, which had been shown to arise from the C_1-transfer pool, was essentially devoid of radioactivity. The value found for C_7 (0.036 C) is slightly higher than anticipated, since this carbon theoretically originates as acetate methyl group. In this case, it is probable that the source of additional activity (as opposed to the values for C_1, C_3, and C_5) stems from partial degradation of the parental compound, 3,5-dimethoxy-2,4-dinitrobenzoic acid, which was heated in order to effect its decarboxylation. The subsequent intermediate in this degradation, styphnic acid was found to have 50% of the initial radioactivity of the pulvilloric acid, which can be accounted for as 3.5 of the assumed 7.0 total labeled carbons. The brompicrin reaction on this intermediate collectively provided C_9, C_{11}, and C_{13}, with a total activity of 0.10 C. It can be concluded by difference, that the average values for the three ring carbons C_8, C_{10}, and C_{12} is 1.13 C. This result is in general agreement with the anticipated acetate–polymalonate biosynthetic route to the backbone carbon chain of this metabolite. Acceptance of these data for the specific activities of the three ring carbons, would suggest that slightly lower specific radioactivities in the carbon atoms of the fatty acyl side-chain portion of pulvilloric acid were found, as opposed to those which are in the alicyclic ring moiety.

With ethyl hexanoate-1-^{14}C as a test percursor, only 0.19% of the initial radioactivity ultimately became incorporated into pulvilloric acid. This is some tenfold less efficiency, when compared with the uptake of label from acetate or from formate, under comparable growth conditions. Partial degradation of the pulvilloric acid isolated in this experiment led to the data presented in Table III. It can be seen that alkaline hydrolysis of this species of labeled pulvilloric acid gave C_{14} with 13% of the total activity in the molecule, or 0.91 C on the basis of 6.1 C remaining in the major 1-(3,5-dihydroxyphenyl)-2-heptanol fragment. Had the hexanoyl fragment become incorporated as an intact unit, this carbon would have been unlabeled. From these results, it is readily apparent that the hexanoate was biologically converted to acetyl units prior to incorporation of its radioactivity into the antibiotic.

Discussion

From the results of the incorporation experiments with acetate-1-^{14}C and formate-^{14}C, pulvilloric acid formation appears closely to follow the pathway which was established by Birch *et al.* (1958), Schwenck *et al.* (1958), and Rodig *et al.* (1966) and their coworkers for citrinin biogenesis. In the case of citrinin formation, evidence has further been adduced by the use of mutant strains of *Aspergillus terreus*, as well as by a study of congeneric substances to citrinin which were produced by *P. citrinum*, that an appropriately methyl-substituted C_{10}-polyketide precursor is first cyclized to a *C*-acetyl-*o*-orsellinic acid, which becomes converted into a dihydroisocoumarin lactol and then to dihydrocitrinone. By analogy, a hypothetical C_{14}-polyketide precursor (Turner, 1966) of pulvilloric acid might be expected to rearrange to a *C*-hexanoyl-*o*-orsellinate and its related heterocyclic structures on the path of biosynthesis. Attempts to detect and identify cometabolites of pulvilloric acid in extracts of the growth filtrates of *P. pulvillorum* ACC 1124, using paper and thin-layer chromatography, were negative. Those substances which were usually detected were the antibiotic itself, and varying amounts of a violet-colored material. This latter mixture probably represents oxidative decomposi-

TABLE II: Distribution of Radioactivity in Pulvilloric Acid from Acetate-1-^{14}C.[a]

Compd or Fragment Counted	Sp Act. (dpm/mmole)	% Act.	No. of Labeled Carbons[b]	
			Theoretical	Found
Pulvilloric acid	2.28×10^7	100	7.0	
1-(3,5-Dihydroxyphenyl)-2-heptanol	2.07×10^7	91	6.0	6.3
$BaCO_3$ (C_{14})	2.81×10^6	12	1.0	0.86
$BaCO_3$ (C_{15})	8.15×10^4	0.36	0	0.03
1-(3,5-Dimethoxyphenyl)-1-heptene	1.79×10^7	78	6.0	5.5
$BaCO_3$ (C_7)	1.19×10^5	0.52	0	0.036
Styphnic acid	1.14×10^7	50	3.0	3.5
Brompicrin ($C_{9,11,13}$)	3.09×10^5	1.4	0	0.10
$BaCO_3$ ($C_{8,10,12}$)			3.0	(3.4)[c]
$BaCO_3$ (C_6)	3.13×10^6	14	1.0	0.96
$BaCO_3$ (C_5)	7.00×10^4	0.31	0	0.02
$BaCO_3$ (C_4)	3.02×10^6	13	1.0	0.93
$BaCO_3$ (C_3)	5.43×10^4	0.24	0	0.02
$BaCO_3$ (C_2)	2.99×10^6	13	1.0	0.92
$BaCO_3$ (C_1)	5.77×10^4	0.25	0	0.02

[a] Cultural conditions identical with those given in the previous table. Sodium acetate-1-^{14}C (0.63 mCi), 44.9 mg was used. Work-up and degradations as described in text of this paper and of part I. [b] Based upon seven acetate units in the parental molecule. [c] Calculated by difference.

tion products of pulvilloric acid. When samples of pulvilloric acid were purified by absorption chromatography on anhydrous magnesium sulfate, this violet-colored complex was retained on the column. Further elution of such chromatograms with solvents more polar than ether did not release any new compounds. It is possible that additional metabolites in this series may be found to accumulate in fermentation beers when mutant strains are made from this particular wild type isolate.

The data which were obtained following the degradation of pulvilloric acid samples produced in the presence of formate-^{14}C and of acetate-1-^{14}C, are not inconsistent with the previously mentioned hypothesis of Turner (1966), in which a linear, methyl-branched polyketide chain (Birch, 1962, 1967) of seven acetate units was proposed as a fairly immediate precursor. The phenomenon of a higher specific activity in the initiating acetate unit, as opposed to those which are derived *via* subsequent acetyl extensions with malonyl-CoA, noted by Birch and his group (*cf.* Birch, 1967, for references) during their earlier studies on the biosynthesis of several polyacetate fungal substances, was not reflected to any degree in the incorporation pattern of acetate-1-^{14}C into pulvilloric acid. The values found for C_2, C_4, and C_6 are particularly reliable, since the starting hexanoic acid and the following lower fatty acids which were obtained from it by Schmidt degradation and permanganate oxidation, were each purified to homogeneity (Tanenbaum and Nakajima, 1969) as evidenced by gas–liquid partition chromatography. These alternating carbons were, within experimental degradative and counting errors, almost identical in their specific activities. The results for this portion of the pulvilloric acid molecule parallel those found (Birch *et al.*, 1962) for rubropunctatin formation from radiolabeled acetate. Here, it was shown that there was little variation in activity from the initiating acetate unit to those further down the carbon chain. Indeed, three of the ring carbons (C_8, C_{10}, and C_{12}) of pulvilloric acid appear to have a slightly higher specific activity than those comprising the alternating active carbons in the fatty acyl side chain. Although the average radioactivity for these carbons is somewhat unreliable since they were ascertained by difference, consideration of these data at face value suggests the origins of pulvilloric acid in two polyacetate generating systems. This notion accords with the finding of Hadfield *et al.* (1967), who degraded a species of rubropunctatin biosynthesized in the presence of diethyl malonate-1-^{14}C, and found that the specific activities of the

TABLE III: Distribution of Radioactivity in Pulvilloric Acid from Ethyl Hexanoate-1-^{14}C.[a]

Compd or Fragment Counted	Sp Act. (dpm/mmole)	% Act.
Pulvilloric acid	2.43×10^6	100
1-(3,5-Dihydroxyphenyl)-2-heptanol	2.11×10^6	87
$BaCO_3$ (C_{14})	3.17×10^5	13
$BaCO_3$ (C_{15})	7.29×10^3	0.30

[a] Cultural, isolation, and degradative conditions identical with those given in the previous tables. Ethyl hexanoate-1-^{14}C (0.54 mCi) was used.

acetate equivalents in the side chain were slightly lower than those of the ring system. In this regard, an essential difference between the formation of pulvilloric acid (and of ascochitine) as compared with citrinin, is that a highly reductive stage analogous to those involved in rubropunctatin and monascorubrin synthesis (Hadfield *et al.*, 1967) or in fatty acid synthesis (Lynen, 1967), must occur. This process can be thought of as taking place in terms of malonyl extensions of a hexanoyl or of a β-ketooctanoyl fragment, which may be biosynthesized by an intermediary chain length fatty acid synthetase. Indeed, β-ketoacyl acyl carrier protein synthetase from *Escherichia coli* will utilize propionyl, butyryl, caproyl, and octanoyl acyl carrier proteins as "starters" for polymalonyl chain extensions (Toomey and Wakil, 1966). An alternative consideration is that the C_{14}-polyketide precursor of pulvilloric acid undergoes selective reductions at the initiating end, with appropriate methylation and cyclization reactions near its terminal end.

Since the isotope distribution pattern found above is suggestive of the first of these two hypotheses, the addition of radioactive β-ketooctanoate as a test substrate for *P. pulvillorum* was first considered. The known metabolic instability of β-keto esters in biological systems caused abandonment of this notion in favor of using hexanoate-1-^{14}C. Because of the possibility of permeability and toxicity difficulties with the free fatty acid (Cochrane, 1958), and in the hope of obtaining enzymatic transfer to the appropriate phosphopantetheine-linked enzyme, the free fatty acid was first transformed into its ethyl ester. The results obtained following introduction of this substance to the growing mold and degradation of the resultant pulvilloric acid, showed that the hexanoate had been broken down to the acetate level prior to its uptake into the antibiotic. This result was not totally unexpected, since Birkinshaw and Gowlland (1962) using butyrate, and Birch (1962) who used acetoacetate and 3,5-diketohexanoate, had recorded similar results in studying secondary metabolite formation. Again, in the case of pulvilloric acid, this negative experiment shed no light on the mechanism of the biogenesis of the *n*-pentyl side chain.

To answer the question of whether such polyketide-derived metabolites which have alkyl side chains, are made first by a process of malonyl extensions of lower fatty acids, or are formed by selective and possibly concerted reductive, dehydrative, and ligative steps at different parts of extended, polyketo acyl precursors, recourse will most probably have to be made toward finding cell-free enzyme extracts which can effect both fatty acid and aromatic formation. Thus, it is known from the work of Light (1965) with doubly labeled acetate, that a close metabolic parallel exists between the formation of aromatic phenols and of higher fatty acids in the *Penicillia*. In addition, such lower oligoketides as 2-methyltriacetic acid (Brenneisen *et al.*, 1964; Acker *et al.*, 1966), triacetic acid (Light *et al.*, 1966), and tetraacetic acid (Bentley and Zwitkowits, 1967) have been isolated as their stable lactones from various *Penicillia* which accumulate tropolone derivatives or phenolic substances. Triacetic lactone accumulation under special circumstances has also been noted in the fatty acid synthetase enzyme complex from *E. coli* (Brock and Bloch, 1966). A previous attempt to relate the fatty acid multienzyme complex (Lynen, 1967) with the polyketide–aromatic synthetic system (Light and Hager, 1968) by seeking the occurrence of 4-methylmyristic acid, the fully saturated fatty acid analog of pulvilloric acid, was not successful (Tanenbaum and Nakajima, 1968). However, indications of regulatory aspects of fatty acid *vs.* polyketide formation in enzyme extracts have been observed by Gatenbeck and Hermodsson (1965) who studied alternariol synthesis, and by Gaucher and Shepherd (1968) who examined orsellinic acid formation. Application of the technics employed by these investigators to compounds of the type exemplified by pulvilloric acid may yet demonstrate an intermeshing of fatty acid synthetase and polyketide–aromatic synthetase multienzyme complexes.

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Effects of Organic Solvents on the Spectrum of Cytochrome *c*[*]

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ABSTRACT: The effects of various alcohols, ethers, and amides on the absorption spectrum of cytochrome *c* have been investigated. A hemoundecapeptide, isolated from a peptic hydrolysate of cytochrome *c*, was similarly studied. (a) Increasing the concentration of organic solvent normally caused an initial increase in the intensity of the Soret peak, which subsequently decreased with further increases in solvent concentration. The maximum slope of a graph of absorbance change against mole per cent of organic solvent, S_M, was used as the criterion for comparing the effectiveness of the organic solvents. (b) The nature of the effects was similar in cytochrome *c* and the hemopeptide, but the magnitude of the change was usually two to three times greater in the hemopeptide. If the increase in Soret absorbance reflects denaturation, this result implies that the structure of the hemopeptide in aqueous solution is not completely random. (c) The addition of electrolytes significantly enhanced the S_M, presumably due to the encouragement of the disruption of ionic bonds in solutions of higher ionic strength. (d) The presence of urea similarly enhanced the S_M. (e) An increase in the length of the hydrocarbon chain of the alcohols and ethers increased S_M. Branching, however, produced a decrease. Hydrophobicity is thus important for conformational changes but accessibility of the solvent hydroxyl group or the interior of the polypeptide chain is also important. (f) The importance of hydrophobicity is also evident from the decrease in S_M which occurs on replacing a carbon by an oxygen or on inserting an oxygen into the chain. When a hydroxyl group replaces a hydrogen atom, the loss of effectiveness is even greater. (g) When chain length and oxygen content were simultaneously increased by adding a carbinol group, the above opposing effects balanced each other. (h) Hydrogen bond disrupting capacity is also important, as determined by comparison of formamide and dimethylformamide. On a volume per cent basis these are of roughly equal effectiveness in enhancing the Soret peak. Thus the increase in hydrophobicity only just balances the loss of hydrogen bonding capacity.

The above results are interpreted as qualitative evidence for contribution of hydrophobic, hydrogen, and ionic bonds to the stability of the native structure of both cytochrome *c* and the hemopeptide.

The effects of organic solvents on macromolecules have recently been studied with a view to estimating the relative importance of hydrophobic, hydrogen, and ionic bonds in stabilizing the preferred conformation. Among the molecules studied are DNA (Herskovits, 1962) and the proteins collagen (Herbage *et al.*, 1968), myoglobin, and α-chymotrypsinogen (Herskovits and Jaillet, 1969), and xanthine oxidase (Dastoli and Price, 1967).

In these studies the organic solvents denatured the protein. Despite the detailed studies of Gordon and Jencks (1963) on albumin, sufficient data are not yet available for derivation of a quantitative relationship between the properties of the solvent and the solvent susceptibility of the protein. Qualitative trends are, however, apparent in these reports. In the case of myoglobin, the more hydrophobic the solvent the greater is its denaturing power. In contrast, hydrogen-bonding capability is of primary importance in the case of albumin, where the hydrophobic character of the solvent contributes little to its effectiveness.

The properties of cytochrome *c* in a nonaqueous environment are of particular interest, since cytochrome *c* is thought to exert its physiological function within the relatively hydrophobic environment of the mitochondrial membrane (Ambe and Crane, 1959). The aerobic oxidation of cytochrome *c* by soluble cytochrome oxidase preparations is accelerated by the addition of lipids (Greenlees and Wainio, 1959) and cytochrome *c* has been shown to form complexes with phospholipids (Reich and Wainio, 1961).

A preliminary report on the three-dimensional structure

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