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THE STRUCTURE OF BIOTIN*

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DURING the past year my associates and I have been working on the structure of biotin and I should like to take this opportunity of presenting to you the results of this study. In 1940, our group at Cornell University Medical College, in collaboration with Dr. Paul György and Catharine S. Rose at Western Reserve, had demonstrated that biotin, the yeast-growth substance which had been isolated by Kögl, was actually identical with vitamin H.[1, 2, 3] Vitamin H was the name which had been given by György to the factor present in liver, yeast and various foods which was capable of preventing the fatal syndrome resulting from the feeding of large amounts of raw egg white, a syndrome found to occur in all species studied. We were thus able to show that biotin was involved in animal metabolism and through this work biotin became recognized as a member of the vitamin B-complex. The full role in nutrition of this newcomer to the group of vitamins is not fully understood, yet there are indications that it may be extremely important. There are now scores of laboratories working on this compound and within the next year or two much light should be thrown on the significance of this vitamin. With the demonstration of the identity of vitamin H with biotin we undertook a study of the chemical nature of this compound and have recorded from time to time some of our chemical findings. We

* A lecture delivered before the New York Section of the American Chemical Society, on October 9, 1942.

[1] P. György, D. B. Melville, D. Burk and V. du Vigneaud, SCIENCE, 91: 243, 1940.

[2] V. du Vigneaud, D. B. Melville, P. György and C. S. Rose, SCIENCE, 92: 62, 1940.

[3] P. György, C. S. Rose, K. Hofmann, D. B. Melville and V. du Vigneaud, SCIENCE, 92: 609, 1940.

have announced for example, that biotin is a cyclic urea derivative[4, 5]; that it contains sulfur in thio ether linkage[4]; that through the oxidation of the compound one can obtain adipic acid;[6] and that after the elimination of the carboxyl group of biotin, adipic acid can no longer be obtained.[7] On the basis of these facts and on consideration of the saturated character of the compound and the empirical formula, we were led to suggest that biotin was a bi-cyclic compound and that there were 5 structures capable of explaining the data up to that time; that is, last January.[8] We have now gone further with the study and have arrived at what we believe with considerable confidence to be the structure of biotin. I propose to confine my presentation to this actual chemical work, foregoing consideration of the biological aspects. I have earlier reviewed the historical side of the problem.[9]

In presenting these structural studies, I would like to pay tribute to the teamwork of the group participating in the work. I would like to acknowledge in particular the splendid contributions which Dr. Melville and Dr. Hofmann have made in this degradation work. Drs. Brown, Kilmer and Armstrong of our group have also made important contributions to the problem in connection with the synthesis of ring sulfur compounds which have helped us to understand certain aspects of biotin chemistry.[10] I wish to acknowledge the cooperation of Mr. Frohring and the Research Staff of the S.M.A. Corporation and Dr. Major and the Research Staff of the Merck Research laboratories for supplies of crystalline material. I would also like to acknowledge the collaboration in a certain phase of the work on desthiobiotin of a group from the Merck laboratories; namely, Drs. Folkers, Wolf, Keresztesy, Harris and Mozingo. I shall mention others of the group in the course of the discussion who have likewise made valuable contributions to the work. Finally I would like to acknowledge the benefit of many fruitful discussions with Professor Hans Clarke, who followed step by step the course of these studies with such great interest.

By chromatographic procedures which we have already described[11, 12] we were able to isolate biotin from liver extracts and from milk concentrates. The compound was isolated as the methyl ester, which by repeated crystallizations from a mixture of methanol and ether was obtained in long, thin, plate-like needles. The ester melted sharply on the hot-stage at 166–167°. This melting point was considerably higher than that reported by Kögl and Tönnis.[13] Subsequently Kögl has reported that his material was impure and he has now reported a melting point which is substantially in agreement with ours.[14] A chloroform solution of the ester showed an optical rotation of $+57°$.

Expressed in terms of vitamin H units the various preparations of purified product that we prepared all consistently yielded, by the yeast-growth method, the high value of 27,000 ($\pm$ 10 per cent.) vitamin H units per mg. (The vitamin H unit is the amount necessary per day for 30 days to cure egg white deficiency symptoms.) Half-maximum growth of the yeast culture was obtained at a concentration as little as 1 part in 1×10^{10}, which indicates the tremendous activity of this material. Direct vitamin H assays of the crystals by Dr. György, carried out with rats by the curative method, were in agreement with this high potency. This means that approximately 0.04γ per day suffices to prevent the fatal syndrome resulting from the egg-white diet employed in the feeding of the rats, truly an amazing potency.

The analytical values we obtained from the pure crystalline compound agreed most closely with the empirical formula of $C_{11}H_{18}O_3N_2S$, which agrees with that given by Kögl. The free biotin was readily obtained by saponification of the ester with cold alkali.[15] Upon acidification of the saponification mixture with HCl, free biotin separated in long, thin needles. The analytical figures pointed to the composition $C_{10}H_{16}O_3N_2S$, which is in good agreement with the composition of the ester. An alkaline solution of the biotin showed an optical rotation of $+92°$. The titration curve run by Dr. Rachele of our laboratory, who likewise carried out all the micro analyses, resembled the titration curve of a simple monocarboxylic acid. The neutral equivalent of 244 obtained from the curve agreed with that expected for a monocarboxylic acid of the empirical formula given. In the yeast-growth assay the free biotin appears to have the same potency per mole as the ester. For some micro-organisms it is necessary, however, to have biotin in the free form and not as the ester.

With the crystalline material available it was pos-

[4] K. Hofmann, D. B. Melville and V. du Vigneaud, *Jour. Biol. Chem.*, 141: 207, 1941.

[5] D. B. Melville, K. Hofmann and V. du Vigneaud, SCIENCE, 94: 308, 1941.

[6] K. Hofmann, D. B. Melville and V. du Vigneaud, *Jour. Am. Chem. Soc.*, 63: 3237, 1941.

[7] K. Hofmann, D. B. Melville and V. du Vigneaud, *Jour. Biol. Chem.*, 144: 513, 1942.

[8] V. du Vigneaud, K. Hofmann and D. B. Melville, *Jour. Am. Chem. Soc.*, 64: 188, 1942.

[9] V. du Vigneaud in Evans, "The Biological Action of the Vitamins," University of Chicago Press, 1942.

[10] G. W. Kilmer, G. B. Brown, M. D. Armstrong and V. du Vigneaud, *Jour. Biol. Chem.*, 145: 495, 1942.

[11] V. du Vigneaud, K. Hofmann and D. B. Melville, *Jour. Biol. Chem.*, 140: 643, 1941.

[12] D. B. Melville, K. Hofmann, E. Hague and V. du Vigneaud, *Jour. Biol. Chem.*, 142: 615, 1942.

[13] F. Kögl and B. Tönnis, *Z. Physiol. Chem.*, 242: 43, 1936.

[14] F. Kögl and L. Pons, *Z. Physiol. Chem.*, 269: 61, 1941.

[15] V. du Vigneaud, K. Hofmann, D. B. Melville and J. R. Rachele, *Jour. Biol. Chem.*, 140: 763, 1941.

sible to obtain preliminary information on the stability and behavior of the compound towards various reagents by inactivation and reactivation experiments using small amounts of material.[16]

In actually tackling the characterization of the functional groups by direct chemical means we first directed our attention towards the nitrogen and oxygen. Two of the three oxygen atoms, of course, had been accounted for by the carboxyl group. Possibility after possibility of how the remaining oxygen and two nitrogen atoms were arranged was eliminated. There is no point, however, in going into the many negative experiments in this direction. It became very puzzling as to what the nature of the nitrogen might be. However, the break came when a cleavage product was obtained after treatment of the biotin with strong barium hydroxide for 20 hours at 140°.[4] This brought about the formation of a new diamino acid which could be isolated in excellent yield. The analysis of the free compound led to the empirical formula $C_9H_{18}O_2N_2S$. It was clear that the split product had lost one carbon and one oxygen and taken up two hydrogens. No loss of anything else occurred. The most logical interpretation we could place on this was the cleavage of a cyclic urea derivative. The hydrolytic cleavage of biotin could therefore be expressed by the following equation.

$$C_8H_{13}S\begin{cases}-COOH\\-NH\\-NH\end{cases}\!\!>C=O \xrightarrow[140°]{Ba(OH)_2} C_8H_{13}S\begin{cases}-COOH\\-NH_2\\-NH_2\end{cases}$$

You will note that the urea structure and the carboxyl group accounted for all the oxygen and the nitrogen, leaving the sulfur to be accounted for. Again many possibilities were eliminated and we suspected that the sulfur was present as a thio ether. A second break came when a crystalline sulfone ($C_{10}H_{16}O_5N_2S$) was obtained by the action of H_2O_2, which led to our recognition that the sulfur was present as a thio ether.[4]

It is obvious that if biotin were a urea derivative and if the barium hydroxide treatment yielded a diaminocarboxylic acid then we should be able to resynthesize biotin from the diaminocarboxylic acid by closing the ring again through urea formation. This we were able to accomplish by treatment of the diaminocarboxylic acid with phosgene[5] as shown in this equation.

$$C_8H_{13}S\begin{cases}-COOH\\-NH_2\\-NH_2\end{cases} \xrightarrow[NaOH]{C(=O)(Cl)_2} C_8H_{13}S\begin{cases}-COOH\\-NH\\-NH\end{cases}\!\!>C=O$$

By this reaction biotin of the same melting point, crystalline form and optical rotation was obtained in 98 per cent. yield. A mixed melting point of the resynthesized biotin with the isolated natural biotin showed no depression. The resynthesized biotin had the same biological activity as the naturally occurring biotin. This evidence proved beyond a shadow of doubt the cyclic urea structure of biotin.

By taking into account the absence of the ethylenic linkage as well as the nature of the functional groups and the ratio of hydrogen to carbon, we were able to arrive at the conclusion that biotin must contain a bicyclic ring system. In two papers of Kögl and coworkers,[14, 17] identical conclusions were arrived at independently with regard to the nature of the functional groups. In addition they claimed to have obtained evidence that the sulfur is present in a ring. They claimed that they were able to cleave a carbon-sulfur bond and the urea ring of biotin sulfone at the same time and still found the 9 carbons and 2 nitrogens with the sulfur. As we have shown[18] this claim was based on an erroneous deduction so that the Kögl data did not afford evidence for a sulfur-containing ring. Both their evidence and ours, independently arrived at, simply showed the nature of the functional groups.

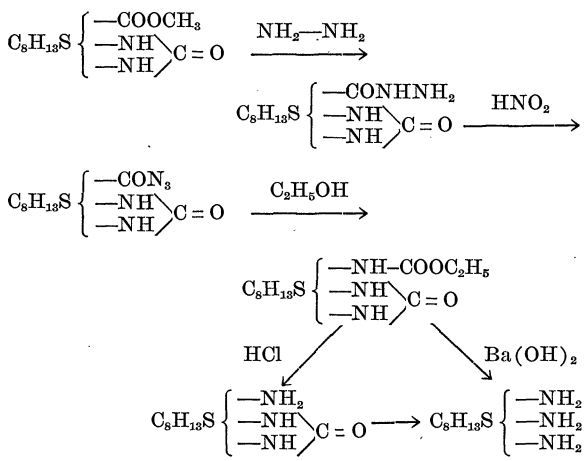
The next question, of course, was how the functional groups were arranged. As a step in this direction we subjected the diaminocarboxylic acid to oxidative degradation to see if we could pick up some characteristic split product. Fortunately we were able to obtain a split product containing 6 carbons in a chain, representing a substantial part of the 9 carbons of the diaminocarboxylic acid.[6] The oxidative degradation was first carried out with alkaline permanganate and later with nitric acid. Out of the mixture of degradation products it was possible to isolate in good yield adipic acid, the 6-carbon dicarboxylic acid. The isolation of the same compound under both these alkaline and acid oxidizing conditions minimized to a great extent the possibility of a rearrangement to an intermediate which could have yielded adipic acid. Thus the consistent formation of adipic acid as an oxidation product of biotin could be interpreted in one of two possible ways. Either biotin contains an aliphatic side chain which is capable of yielding adipic acid; or else the adipic acid has its origin in a cyclic structure which is cleaved by the oxidation. In the first case one of the carboxyl groups of the adipic acid must be the carboxyl group originally present in biotin, and it should therefore be possible, by the oxidation of a derivative of the diaminocarboxylic acid in which the carboxyl group has been eliminated, to decide between the two alternatives. After several

[16] G. B. Brown and V. du Vigneaud, *Jour. Biol. Chem.*, 141: 85, 1941.

[17] F. Kögl and T. J. de Man, *Z. Physiol. Chem.*, 269: 81, 1941.

[18] D. B. Melville, K. Hofmann and V. du Vigneaud, *Jour. Biol. Chem.*, 145: 101, 1942.

attempts by other methods the objective was achieved by a Curtius degradation.[7] In this way the carboxyl group was replaced by an amino group. Biotin methyl ester was converted to the hydrazide, from which the azide was obtained by treatment with nitrous acid. The azide was transformed into the corresponding ethyl urethane. The hydrolysis of the urethane was performed in two ways as indicated in these equations.

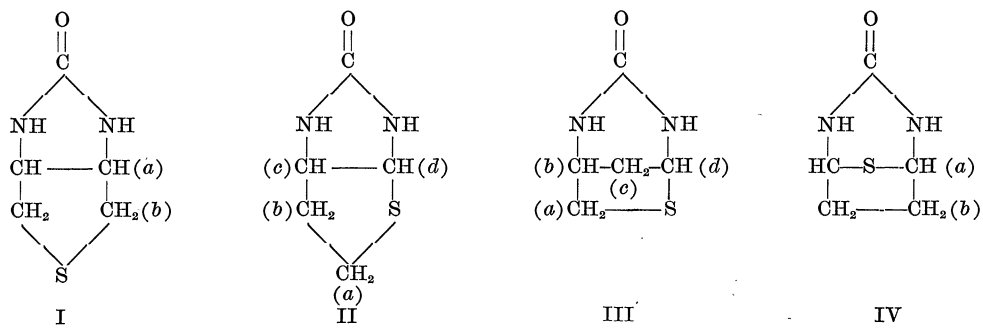


The triamine was subjected to the same oxidation procedures which we employed for the oxidation of the diaminocarboxylic acid. After preliminary experiments, 50 mg of the triamine sulfate were oxidized with potassium permanganate under the same conditions employed in the oxidation of the diaminocarboxylic acid. No trace of adipic acid could be detected in the ether-soluble oxidation products, although the amount of adipic acid which might have been formed from the relatively large amount of triamine used would have made its isolation and identification comparatively easy. The absence of adipic acid in isolable amounts among the oxidation products of the triamine therefore afforded substantial evidence that one of the carboxyl groups of the adipic acid formed by oxidation of the diaminocarboxylic acid is identical with the original carboxyl group of biotin. This means in effect that the 6-carbon, straight chain moiety giving rise to adipic acid upon oxidation is not present in biotin as a cyclic structure, but indicates the presence of an aliphatic acid side chain in biotin which is capable of yielding adipic acid on oxidation.

With all the foregoing data we were in position to list the possible structures which would fit these data. The most logical interpretation of the adipic acid data as a whole was that biotin contained a normal valeric acid side chain. The adipic acid would arise then from this side chain plus one carbon in the ring which was so linked that on oxidation it could give rise to a carboxyl group. With this deduction along with the other chemical data we had we could write, on the basis of 5 or 6 membered rings being present, only the structures indicated by formulas I, II, III and IV, with a valeric acid side chain at the positions indicated.[8]

You will note, however, that formulas II, III and IV have sulfur and nitrogen attached to the same carbon. We felt that the remarkable stability of the diaminocarboxylic acid towards strong hydrolytic agents rendered very unlikely structures where nitrogen and sulfur were attached to the same carbon. Such compounds described in the literature are unstable to strong alkali. As we stated in the preliminary note, formula I with either the side chain in position (a) or position (b) was the most likely formula for biotin.

In order to keep absolutely within the bounds of our data we had to grant another possibility although it seemed to us less likely. It was theoretically possible that the adipic acid might arise from the decarboxylation of a malonic or α-substituted β-keto acid arising during the oxidation, in which case a butyric rather than a valeric acid side chain might be present.

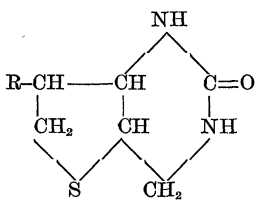


Side Chain = $CH_2CH_2CH_2CH_2COOH$

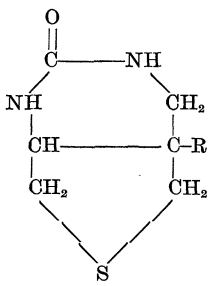
On the basis of such a construction we could arrive at 3 additional structures, as shown in formulas V, VI and VII.

At this stage we therefore had before us 5 structures which we felt were the only ones which could possibly explain the chemical evidence which we had so far adduced. As we pointed out[8] all the formulas with the exception of formula Ia involved, in the

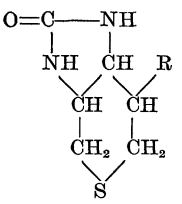
formation of adipic acid, the oxidative cleavage of a carbon-sulfur bond. As we stated it, formulas Ib, V, VI and VII involved the assumption that the carbon atom attached to the sulfur and proximal to the side chain would be oxidized to a carboxyl group. We pointed out that if this assumption were invalid then only structure Ia remained. We did not rule out this assumption. In other words we considered these formulas involving the splitting of the carbon-sulfur bond as entirely possible and our further work was based on the allowance of that assumption. This left us then at this stage with 5 possible structures for biotin, with preference for the fused 5-membered rings with a valeric acid side chain. We had hoped that x-ray data might aid us in ruling out some of the structures. Dr. Fankuchen was kind enough to make



V



VI



VII

R = $CH_2CH_2CH_2COOH$

such an analysis of biotin, but felt that the x-ray data did not warrant a decision on which structure was the more likely in the presentation of his x-ray data.[19] Since our determination of the structure by chemical means he has obtained evidence in favor of it from a study of the x-ray pattern of biotin sulfone.

In the chemical attack it is obvious that an important step would be the establishment of whether the urea ring was 5- or 6-membered, or to put it another way, whether the diaminocarboxylic acid derivable from biotin was a 1,2 or 1,3 diamine. The ring closure with phosgene could not decide between these 2 possibilities and we therefore searched for a ring closure for the diaminocarboxylic acid which could decide between a 1,2 and 1,3 diamine. This was accomplished by recourse to the formation of a derivative of the diaminocarboxylic acid with phenanthrenequinone.[20] While it is well known that many 1,2 diamines will condense with phenanthrenequinone, there is no evidence that 1,3 diamines form a ring structure with this reagent. The diaminocarboxylic acid when treated with phenanthrenequinone yielded a condensation product melting at 202–204° which crystallized in pale yellow needles. The analytical values for the compound agreed somewhat more closely with the composition of the quinoxaline rather than the dihydroquinoxaline derivative. The red color which was obtainable on treatment of the condensation product with sulfuric acid was in favor of this.

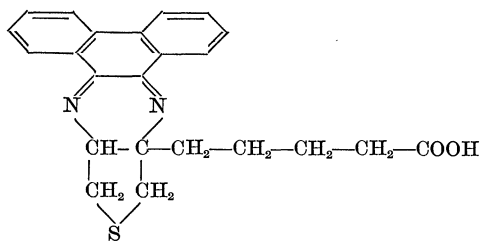
The formation of the derivative with phenanthrenequinone indicated strongly if not proved that the diaminocarboxylic acid is a 1,2 diamine and that therefore biotin possesses a 5-membered urea ring. This is in contradiction to the suggestion of Kögl and Pons[14] that biotin is a 6-membered cyclic urea derivative. The evidence on which they based this suggestion was simply the comparative stability of 5- and 6-membered cyclic urea derivatives toward hydrolysis. The demonstration that the diaminocarboxylic acid was a 1,2 diamine and that biotin therefore contained a 5-membered urea ring eliminated two of the five structures which we have been discussing, namely, those containing the 6-membered urea ring—that is, structures V and VI. The diaminocarboxylic acid Ia, Ib or VII could form a phenanthrenequinone derivative.

As indicated earlier, the behavior of the phenanthrenequinone derivative of the diaminocarboxylic acid aroused the suspicion that we might have obtained the quinoxaline rather than the dihydroquinoxaline derivative from the reaction of the phenanthrenequinone with the diaminocarboxylic acid, *e.g.*, structure Ia could form a dihydroquinoxaline (formula VIII) but would not be expected to yield the dehydrogenated derivative. On the other hand, the two remaining structures, Ib and VII, can give the dehydrogenated derivative since both carbon atoms bearing the amino groups carry hydrogen atoms. For example, structure Ib can yield a quinoxaline as shown in formula IX.

In order to settle definitely whether or not the derivative obtained from the diaminocarboxylic acid was the dihydroquinoxaline or the more fully aromatic quinoxaline, we asked Dr. Hugh H. Darby at the College of Physicians and Surgeons, Columbia

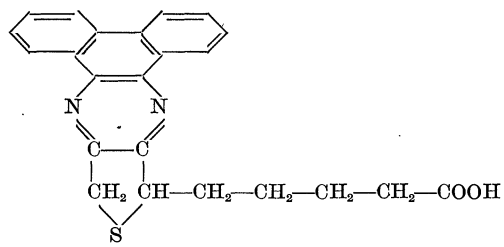
[19] I. Fankuchen, *Jour. Am. Chem. Soc.*, 64: 1742, 1942.
[20] K. Hofmann, G. W. Kilmer, D. B. Melville, V. du Vigneaud and H. H. Darby, *Jour. Biol. Chem.*, 145: 503, 1942.

University, to examine the ultraviolet absorption spectrum of the compound and compare it with the spectra of the dihydrodibenzoquinoxaline and dibenzoquinoxaline derivatives of 3,4-diaminotetrahydrothiophene, which we had synthesized (formulas X and XI).



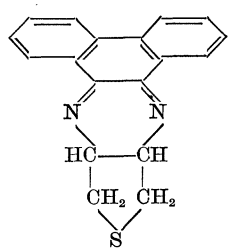
Formula VIII

One would expect a great difference in absorption spectra between these two forms, for one of them, that is the quinoxaline, is more fully aromatic. As we expected, the absorption spectra of these compounds were distinctly different. It was hoped that the absorption curve of the condensation product of the

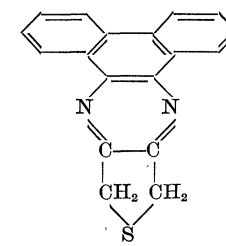


Formula IX

diaminocarboxylic acid from biotin with phenanthrenequinone would show the characteristics of one or the other of these curves. We found, in fact, that the absorption curve of the derivative from biotin was almost identical in form with that of the oxidized, or quinoxaline, derivative from the 3,4-diaminotetrahydrothiophene, and bore little resemblance to the curve of the dihydroquinoxaline derivative. This is a very strong indication that the derivative formed from phenanthrenequinone and the diaminocarboxylic acid from biotin is a dibenzoquinoxaline, and not a dibenzodihydroquinoxaline, derivative. Thus strong evidence was afforded against structure Ia, which left structures Ib and VII still under consideration.



Dihydrodibenzoquinoxaline derivative
Formula X



Dibenzoquinoxaline derivative
Formula XI

The evidence to decide between these has been obtained in two different ways, one by direct proof and another an indirect approach, beautifully confirming each other and pointing without equivocation to the formula Ib. One was in collaboration with the Merck group I mentioned, and the other by our own group.

The indirect proof of the sulfur ring system has resulted from a collaborative investigation with the group from the Merck Research Laboratory. Dr. Mozingo of their laboratory discovered a very ingenious reaction for removing sulfur from organic sulfides with Raney nickel, in which the sulfur was replaced with hydrogen. They found, for example, that treatment of benzoyl methionine yielded benzoylaminobutyric acid, and that the phenyl ureido derivative of methionine gave the corresponding derivative of aminobutyric acid. Still other compounds were studied and it was thought that it might be useful in removing sulfur from biotin and replacing it with hydrogen.

Dr. Donald Wolf applied this reaction in my laboratory to biotin methyl ester and obtained a product containing the same number of carbon atoms and two added hydrogens and no sulfur.[21] This definitely established the cyclic nature of the sulfide group. This "desthiobiotin" was hydrolyzed to a diamino acid. The desthiodiamino acid derivable from structure Ib possesses only one C-methyl group as indicated in formula XII, whereas that derivable from structure VII (formula XIII) possesses two. A Kuhn-Roth C-methyl analysis showed the presence of one. This result was in favor of structure Ib.

More positive characterization was established by oxidative cleavage. It can be seen that oxidation of the diamine should yield pimelic acid, the 7-carbon dibasic acid, if Ib were correct, but α-methyl adipic acid should be formed if VII were correct. Alkaline periodate oxidation yielded pimelic acid, which indicated that the diamino acid was the diamino pelar-

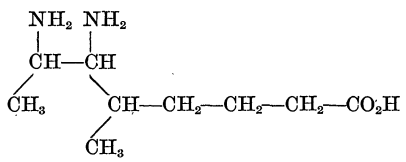
[21] V. du Vigneaud, D. B. Melville, K. Folkers, D. E. Wolf, R. Mozingo, J. C. Keresztesy and S. A. Harris, *Jour. Biol. Chem.* (in press).

gonic acid. The pimelic acid was identified as such and as its di-p-bromphenacyl ester by comparison with authentic samples of each.

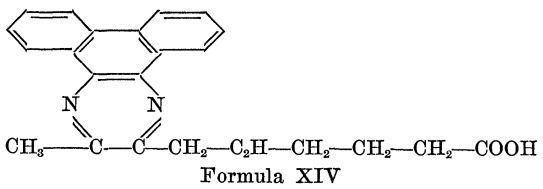
While this work was underway Dr. Folkers and Dr. Harris synthesized the diamino pelargonic acid in the Merck Laboratory. The difficulty we faced, of course, was of comparing the desthiobiotin diaminocarboxylic acid prepared from optically active biotin with the racemic synthetic compound, a difficulty increased by the fact that partial racemization had

Formula XII

apparently occurred in the Mozingo reaction. It occurred to us that these difficulties could be circumvented by preparing the quinoxaline derivatives of both the compound derived from biotin and the synthetic product. As you can see from formula XIV the quinoxaline derivative should possess no asymmetric carbons.



Formula XIII

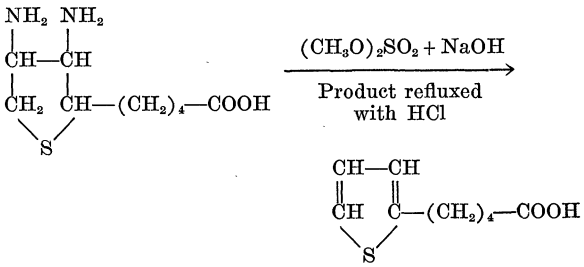


Formula XIV

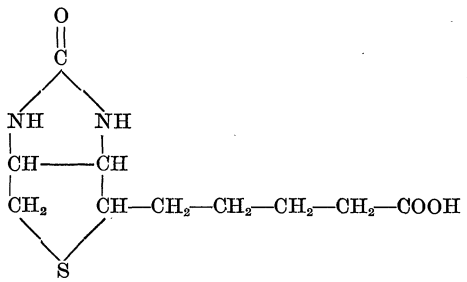
In this way a comparison could readily be made. Consequently Dr. Harris in the Merck Laboratory prepared the quinoxaline derivative of the synthetic compound. Dr. Melville prepared the quinoxaline derivative of the desthiobiotin diaminocarboxylic acid derived from biotin and found that both melted at 186–187° and also that a mixture of the two derivatives showed no depression of the melting point. Thus the desthiobiotin diaminocarboxylic acid was identified as ζ,η-diamino-pelargonic acid.

While this collaborative work was in progress we were also continuing in our laboratory another line of attack we had had underway to obtain direct evidence of the nature of the sulfur ring by keeping the sulfur intact. We felt if our formula Ib were correct we ought to be able to obtain a thiophene derivative from it by degradation, and this we could meet by synthesis. We were convinced that we should be able to obtain δ-(α-thienyl)-valeric acid from the diaminocarboxylic acid. We were so convinced that while the degradation work was progressing we synthesized the compound to have it available for comparison. Dr. Moyer of our laboratory collaborated with Dr. Melville and myself on this phase of our work.

Various attempts were made to bring about the desired decomposition. This was finally accomplished through the decomposition of the methylated diaminocarboxylic acid as shown in the following equation.[22]



The compound obtained by this degradation was compared with the sample of synthetic δ-(α-thienyl)-valeric acid. The synthetic compound was prepared by the condensation of thiophene and glutaric anhydride to give a keto acid which was reduced by a Clemmenson reduction to the thienyl valeric acid. This reaction is analogous to that used by Fieser in the synthesis of thienyl butyric acid in which succinic anhydride was condensed with thiophene. The position of the side chain in our synthetic compound was proved by oxidation of the keto acid to α-thiophenic acid. The synthetic δ-(α-thienyl)-valeric acid was found to be identical with the isolated compound, and thus by this step the five-membered sulfur ring with a valeric acid side chain attached to the carbon alpha to the sulfur was directly demonstrated. On the basis of this and the other data I have presented in this lecture we feel justified in concluding that the structure of biotin is that represented by formula Ib, as shown here in full in formula XV.



Formula XV

[22] D. B. Melville, A. W. Moyer, K. Hofmann and V. du Vigneaud, *Jour. Biol. Chem.* (in press).