# SCIENCE

# Trail of Sulfur Research: from Insulin to Oxytocin

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The work on biochemically important sulfur compounds, cited in the Nobel award, formed a part of a trail of "sulfur research" that began approximately 30 years ago with a study of the sulfur of insulin and encompassed recently the first synthesis of a polypeptide hormone. The latter sulfur-containing hormone, oxytocin, is the principal uterinecontracting and milk-ejecting hormone of the posterior pituitary gland. It might therefore be of interest to unravel some of the stepwise evolvement of our researches from insulin to oxytocin and to bring into focus the background of the research findings and the thinking behind the researches.

I have had the pleasure of following this trail of research in the company of a group of graduate students and postdoctoral associates, without whose loyal and effective collaboration the trail that I am going to describe could not have been worked out.

This trail becomes apparent only in retrospect. Obviously, I did not start out to study sulfur as my life's work. And yet, as I look back over the trail of many years, I encounter the fact that this thread of sulfur has been the thread of continuity running through practically all of my research endeavors.

I find it intriguing to contemplate how one starts out on a trail of exploration in the laboratory, not knowing where one is eventually going—starting out, to be sure, with some immediate objective in mind but also having a vague sense of something beyond the immediate objective toward which one is striving. The thrill of this kind of research, albeit at a sublimated level, is analogous, I am sure, to the thrill that explorers like the Vikings-of-old-experienced in breakingthrough the confines of the known world.

As one looks back on a trail of research, one sees that the continuity is sometimes greater than he may have imagined at the time. As I look back, it seems almost inevitable that I should have proceeded from the pancreatic hormone, insulin, to the posterior pituitary hormone, oxytocin, via sulfur. Again, in looking back, I now realize that there were times when we stopped to work out more fully the chemistry of other sulfurcontaining compounds, such as cystine, homocystine, and methionine, and to study their metabolic significance in transulfuration and transmethylation. These researches diverted us into interesting bypaths. However, strangely enough, as I shall try to bring out, many of these experiences, particularly the cystine peptide work and the knowledge of certain reactions involving sodium and liquid ammonia that we gained along the route, were vital when we came to study the posterior pituitary hormones.

#### Insulin

Where did the sulfur trail start? I think it started at the University of Illinois, where my first teacher in biochemistry was the late H. B. Lewis, who was extremely enthusiastic about sulfur. It was his enthusiasm that undoubtedly aroused my interest in the biochemistry of sulfur compounds.

In my work in organic chemistry at

that same university with C. S. Marvel, I developed a strong interest in the relationship of organic chemical structure to biological activity. The continuing interest in this relationship has also influenced greatly the direction of this trail of research. The work I did with Marvel was concerned with the synthesis of compounds which we hoped would have a local anesthetic effect accompanied by an epinephrinelike effect. As I look back over the trail, I find this theme—the relation between biological activity and organic chemical structure—recurring again and again.

My interest in insulin was initiated through a lecture given by W. C. Rose, who succeeded Lewis as professor of biochemistry at Illinois. On his return from a meeting in Toronto in 1923, he gave an account of the exciting discovery of insulin by Banting and Best. I well recall the thrill of listening to Rose and my curiosity about the chemical nature of a compound that could bring about the miracles he described. Little did I know at that time that insulin would eventually turn out to be a sulfur compound.

Some 2 years later, I received an invitation from J. R. Murlin at the University of Rochester Medical School to come and work on the chemistry of insulin in his department, a department that was devoted mainly to endocrinology and metabolism. The chance to work on the chemistry of insulin transcended all other interests for me, and I accepted Murlin's invitation.

While I was at Rochester, I became intrigued with the fact that all of our preparations contained sulfur, and most of my efforts during the next 2 years were devoted to studying the sulfur of these insulin preparations. From these studies, I came to the conclusion that the sulfur was present in the form of the disulfide linkage and that insulin was most likely a derivative of the amino acid, cystine, and the suggestion was made that the cystine in insulin was linked to the rest of the molecule by peptide linkages (1).

The following year, while working in Abel's laboratory at Johns Hopkins University, I took up the isolation of cystine from crystalline insulin, because the conclusive proof of the presence of cystine in insulin had to rest on the isolation of cystine in pure form. This isolation was eventually accomplished (2). As I con-

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tinued work at Johns Hopkins on insulin in collaboration with Jensen and Wintersteiner, we could find nothing but ordinary amino acids and ammonia in acid hydrolyzates of insulin (3, 4).

The presence of cystine in insulin naturally brought many questions to mind. One of the first questions that occurred to me was whether various combinations of cystine with other amino acids in peptide linkage might affect the lowering of blood sugar. It was then realized that as yet no peptide of cystine that was linked through the carboxyl group of cystine had been prepared. No method was available at the time for the synthesis of this type of peptide. Therefore, while the researches on insulin were continued over the next several years, parallel studies were carried out with several graduate students on the synthesis of peptides of cystine.

### **Cystine Peptides**

Although the peptides we eventually made did not have hypoglycemic activity, the work we did on them gave us valuable experience in the synthesis of cystine peptides and also led to a synthesis of the biologically important compound, glutathione, a tripeptide of glutamic acid, cysteine, and glycine. This work on cystine peptides led to the development by us of several reactions involving sodium and liquid ammonia, which, almost 20 years later, played a vital role in our synthesis of oxytocin. One of these reactions was the removal of a carbobenzoxy group by sodium in liquid ammonia, and another was the utilization of a benzyl group to cover the sulfur of cysteine during certain steps of synthesis and its final removal by means of sodium in liquid ammonia.

As is well known, Bergmann and

Zervas introduced in 1932 the now classical method of protecting an amino group during the course of peptide synthesis with what they called a carbobenzoxy group (5). Their procedure led to the carbobenzoxy derivative of the peptide, and the carbobenzoxy group was removed by catalytic reduction with hydrogen. However, their procedure did not lend itself to the preparation of cysteine or cystine peptides.

Two years after the appearance of the Bergmann-Zervas method, it occurred to me that the carbobenzoxy group might possibly be cleaved from the amino group of cystine by reduction with sodium in liquid ammonia. If so, a convenient method for the synthesis of cystine peptides might result. In work with Audrieth and Loring (6), we had already prepared cysteine from cystine by this method of reduction.

The reduction of dicarbobenzoxycystine was therefore attempted in work with Sifferd (7). The compound was dissolved in liquid ammonia, and sodium was added until a permanent blue color was obtained. After evaporation of the ammonia and subsequent oxidation of the cysteine by aeration of the slightly alkaline solution, cystine was obtained in almost quantitative yield.

In our earlier work on the preparation of cysteine, it had occurred to us that it might be possible to benzylate the sulfhydryl group of cysteine by adding benzyl chloride to the liquid ammonia solution of the sodium salt of cysteine produced by the reduction of cystine with metallic sodium. An excellent yield of S-benzylcysteine was obtained. Although the latter reaction was carried out in 1930 with Loring and Audrieth, it was not until sometime later that the possibility of cleaving a benzyl thio ether by this same means occurred to us in our work with Sifferd. S-Benzylcysteine was cleaved to



Fig. 1. A synthesis of glutathione.

cysteine in liquid ammonia with metallic sodium, cystine being isolated after oxidation (7).

This same reductive procedure was also applied to the preparation of cystinylbisglycine from dicarbobenzoxycystinybisglycine and of cysteinylglycine from S-benzylcysteinylglycine (8).

#### Glutathione

The effectiveness of these reactions impressed us with their potentialities as possible key reactions for a synthesis of glutathione, the structure of which was believed, through the work of Hopkins (9) and of Kendall, Mason, and McKenzie (10) to be  $\gamma$ -L-glutamyl-L-cysteinylglycine. If we could synthesize N-carbobenz-oxy- $\gamma$ -glutamyl-S-benzylcysteinylglycine, we felt that its reduction with sodium in liquid ammonia should yield glutathione.

This approach to glutathione would have a particular advantage in that the sulfhydryl group would be covered by a benzyl radical up to the final step, and thus, during the course of the various reactions that might be employed up to this point, the likelihood of partial oxidation of the sulfhydryl group and its attendant difficulties would be eliminated.

In work with Miller (11), we were able to obtain this desired intermediate by the coupling of suitable derivatives of the three amino acids involved. As is shown in Fig. 1, reduction of this intermediate with sodium in liquid ammonia gave glutathione, which, upon isolation in crystalline form, was shown to be identical with the natural product. Our synthesis followed shortly after the first synthesis of glutathione by Harington and Mead by a somewhat different approach (12).

While this synthesis was going on, we continued our work on insulin, mainly along two lines. The first was concerned with determining whether the cystine content accounted for all of the sulfur; and the second, with the behavior of insulin upon reduction of the disulfide groups. After some 7 years of work, we finally were able to account for the sulfur of insulin entirely on the basis of cystine (13), and we could obtain no evidence for any sulfur compound other than cystine in insulin (14, 15).

The study of the reduction of insulin led directly to our work on the posterior pituitary hormones. With such a gentle reducing agent as cysteine or glutathione acting at room temperature and at a neutral pH, insulin became inactivated, reduction of the disulfide linkages being undoubtedly the cause of the inactivation (16-18). Reoxidation did not restore activity.



Fig. 2. Separation of amino acids from a hydrolyzate of oxytocin (upper chromatogram) and from an artificial mixture of amino acids simulating the composition of oxytocin (lower chromatogram). Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1N HCl followed by 2:1 *n*-propyl alcohol-0.5N HCl.

#### **Posterior Pituitary Hormones**

The work on insulin aroused our interest in other protein or proteinlike hormones. We turned to the examination of oxytocin, the uterine-contracting hormone, and vasopressin, the blood-pressure-raising hormone, of the posterior pituitary gland. There were some indications in the literature that these hormones might be polypeptidelike substances of lower molecular weight than insulin. Furthermore, there was evidence that partially purified preparations of these hormones contained sulfur, but the nature of the sulfur was unknown. We thought it would be interesting to investigate these hormones in comparison with insulin, and in 1932 we made some preliminary explorations on these hormones. In this discussion I will confine my attention mainly to oxytocin, with only occasional reference to vasopressin.

Kamm and Grote of Parke, Davis and Company kindly placed at our disposal some of their partially purified oxytocin, and we were able to show that, upon hydrolysis, the samples contained approximately 9 percent cystine (19). Of course, we could not tell at that time whether the cystine was present in the hormone or in the impurities. Nevertheless, in the work with Sealock (20), we decided to treat the partially purified oxytocin with cysteine and find out whether this hormone lost its activity like insulin. Much to our surprise, the oxytocic activity remained. Oxidation, by aeration of an aqueous solution until the sulfhydryl test was negative, did not cause loss of activity. The question then occurred: Had we really reduced the hormone by the cysteine treatment? It appeared possible to us that if the hormone were a disulfide and had been reduced, treatment with benzyl chloride might cover the sulfhydryl group with a benzyl radical and inactivation might take place. When the reduced oxytocin preparation was treated with benzyl chloride, inactivation did result. On the other hand, treatment of the nonreduced material with benzyl chloride did not cause inactivation. These results made us fairly certain that the oxytocic principle contained sulfur in the form of a disulfide linkage (20).

We also investigated the behavior of the vasopressin preparation on cysteine treatment and found it quite parallel to that of the oxytocin preparation. This aroused in us the desire to see what the pure compounds themselves would be like. What manner of compounds were they? Were they, like insulin, also simply made up of amino acids and ammonia? Since we had reason to believe that they were smaller molecules than insulin, it seemed to me that they might lend themselves to an organic chemical approach. If we could isolate them, we thought we might be able to work out their structure and perhaps synthesize them.

#### Isolation

The purification was a slow process, for the amount of active principles in the gland is extremely small, the principles are unstable, and the bioassays involved are very time-consuming. Hundreds of thousands of hog and beef glands were used during the course of the investigations. Up to the time of World War II, we made considerable progress in collaboration with Sealock, Irving, Dyer, and Cohn on the purification of the principles, mainly through electrophoretic techniques, and learned much about the behavior of the hormones (21-26).

We laid aside the problem during the war for certain assignments, particularly on penicillin, but thereafter the isolation of oxytocin was undertaken in collaboration with Livermore (27). Since the countercurrent distribution technique developed by Craig (28) for the purification of organic compounds had played a helpful role in our isolation of synthetic penicillin, we naturally thought of using countercurrent distribution on partially purified oxytocin fractions, prepared by the method of Kamm and coworkers (29). The source material for preparation of the oxytocin fractions was a commercial extract provided by Kamm of Parke, Davis and Company. The countercurrent distribution between 0.05percent acetic acid and secondary butyl alcohol proved to be highly effective. We obtained a fraction that appeared to behave like a pure compound by this criterion, and, through application of the elegant starch-column chromatographic method of Moore and Stein (30), we were able, with Pierce (31), to show that an acid hydrolyzate of oxytocin consisted of eight amino acids and ammonia.

It was now of importance to determine whether, starting from the glands themselves, material of the same potency and properties would be obtained. Therefore the oxytocin was isolated from lyophilized posterior lobes of beef pituitary glands (32). A preparation was obtained that had approximately the same distribution curve and the same potency as the preparation obtained from the concentrate. The two preparations likewise showed the same amino acid composition. The chromatogram of the amino acids in the hydrolyzed oxytocic material from beef posterior pituitary lobes is shown in Fig. 2. The amino acids were present in a molar ratio to each other of 1 to 1, and the molar ratio of ammonia to any one amino acid was 3 to 1. Molecular weight determinations indicated a molecular weight in the neighborhood of 1000.

The sulfur content of oxytocin could be entirely accounted for by cystine. Knowing the cystine content of the purified hormone, it becomes evident, from the sulfur content of all the prewar preparations, including those of Kamm and coworkers and our preparations, that none of these preparations could have been more than 50-percent pure, regardless of the unitage ascribed to them on an arbitrary basis by reference to a standard powder. No greater purity than 50 percent could be attributed to these early preparations, even if the sulfur of the preparations were due entirely to the presence of oxytocin, which is somewhat unlikely, since sulfur-containing impurities may well have been present.

Further countercurrent distribution of the purified oxytocin involving 1000 transfers resulted in no change in composition, and this work (33) culminated in 1952 in the isolation of a crystalline flavianate of oxytocin with Pierce, the first crystalline derivative of this hormone to be isolated.

It is of interest that an oxytocic fraction was also obtained from hog posterior pituitary glands that had a distribution curve approximately the same as that from beef glands (33). In addition, the oxytocin obtained from the hog pituitary had the same amino acid composition and potency as that obtained from beef.

During the course of these studies on the oxytocic hormone, the pressor hormone, vasopressin, was also isolated from beef glands and shown to contain six of the same amino acids as oxytocin. In place of the leucine and isoleucine in oxytocin, vasopressin contained phenylalanine and arginine.

#### **Biological Effects**

With the isolation of what appeared to be the pure hormones and the establishment of their composition, we were for the first time in a position, on a chemical basis, to be quite certain that the oxytocin was free of vasopressin, and therefore it was possible to ascertain the biological effects of oxytocin itself.

Before going into this, it might be well to mention a few of the biological activities that have been attributed to the posterior pituitary gland. I would recall to you that it was just 60 years ago that the first biological effect of the pituitary gland was discovered by Oliver and Schäfer (34). They found that extracts of the pituitary when injected into mammals raised their blood pressure—the pressor effect. Howell showed a few years later that this activity resided in the posterior lobe (35). Since that time, other biological activities of posterior pituitary extracts were noted, particularly the uterine-contracting, or oxytocic, effect by Dale in 1906 (36); the milk-ejecting effect by Ott and Scott in 1910 (37); the blood-pressure-lowering effect in birds the avian depressor effect—by Paton and Watson in 1912 (38); and the inhibition of urine excretion in man, the antidiuretic effect, by von den Velden in 1913 (39).

To determine the biological effects of the purified oxytocin, it was assayed for avian depressor effect against a standard powder according to the method of Coon as described in the United States Pharmacopeia (40) and found to possess this activity to the extent of 450 to 500 units per milligram (33). In addition to the avian depressor effect, the oxytocin was found to have the same potency, relative to the standard powder, in bringing about contractions of the isolated rat uterusthe uterine-contracting activity. The oxytocin also showed the same potency (450 to 500 units per milligram, relative to the standard powder) in bringing about

the ejection of milk. This milk-ejecting activity of oxytocin was demonstrated by tests of our purified material in sows by Whittlestone (41), in rabbits by Cross and van Dyke (42), and in recently parturient women, the latter testing having been carried out in a collaborative study with Douglas, Nickerson, and Bonsnes of the department of obstetrics and gynecology at Cornell University Medical College (43).

We thought at first that oxytocin was devoid of pressor and antidiuretic activity. However, we placed at van Dyke's disposal samples of our purified oxytocin, which he and his colleagues assayed by refined techniques. They found 7 units of pressor and 3 units of antidiuretic activity per milligram. These activities have been confirmed qualitatively and quantitatively with our synthetic oxytocin, so there is no longer any question that they are inherent properties of the oxytocin molecule (44). It might be mentioned that vasopressin, in addition to its pressor and antidiuretic effects (500 to 600





Fig. 4. Benzylated derivative of reduced oxytocin.

units per milligram, relative to the standard powder), also possesses avian depressor, uterine-contracting, and milkejecting activity, but the potency of vasopressin with respect to the latter three activities is only a fraction of the potency of oxytocin.

#### Structure

With the purified oxytocin at hand and its composition established, we then turned to the problem of how the component amino acids were linked. Of course, there were many structures that could be written involving the eight amino acids and ammonia. The greatest difficulty in the degradative work was the scarcity of material. To obtain enough purified hormones was truly a prodigious task, as has already been mentioned. The various degradative steps were perforce carried out on milligrams of material, and, in most instances, the methods had to be adapted to this scale.

Since I am attempting in this presentation to focus attention on the synthesis of oxytocin in relation to this trail of sulfur research, I shall not present the studies involving a variety of procedures in our gradual elucidation of the structure of the hormone. These researches over the course of several years with Pierce, Mueller, Turner, Davoll, Taylor, and Kunkel (45-50), and the final decisive experiments with Ressler and Trippett (51, 52) on the cleavage of performic acid-oxidized oxytocin with bromine water and on the partial hydrolysis and identification of peptide fragments, brought us to a clear-cut concept of the structure of oxytocin, a new type of cyclic polypeptide amide shown in Fig. 3 (52, 53). Although this structure was the only one that we could arrive at through the rationalization of our data, we felt that proof of this structure by synthesis of the compound was mandatory because of certain assumptions involved in postulating it.

It is of considerable interest that Tuppy, on the basis of data we had published along with some data of his own, arrived at the same structure independently (54). Tuppy's proposal was based on the data from our laboratory on composition, molecular weight, terminal groups-as worked out by our use of the Sanger dinitrophenyl end-group procedure (55)—and on the cyclic structure involving the disulfide linkage that we had established, along with his independent studies on the sequence of amino acids involving partial hydrolysis with acid and with an enzyme. The interpretation of the data and the assumptions made were quite parallel in both laboratories.

As I have already mentioned, we felt 1 JUNE 1956

that proof of the structure that we had arrived at had to be adduced by synthesis-that is, of course, if the synthesis were within the range of possibility. I might point out that the synthesis of this structure meant the synthesis of an octapeptide of eight different amino acids, one of them being cystine. In addition, this desired octapeptide contained three amide groupings and was a cyclic polypeptide for which no known synthetic methods were available-a cyclic pentapeptide with a tripeptide side chain. To add to the challenge was the fact that we knew the final product was relatively unstable and easily inactivated. Gentle means would therefore have to be employed.

## Background of Synthetical Approach

The clue to approaching a synthesis of this compound rested on our work with Sealock, carried out in the 1930's, on the reduction of oxytocin and the oxidation of the reduced oxytocin without appreciable inactivation at either step, which has already been discussed (20). On the basis of our postulated structure for oxytocin (Fig. 3), the reduction of oxytocin and subsequent oxidation could be interpreted as involving the opening and closing of the 20-membered ring. The reduced oxytocin would then have a linear structure containing two sulfhydryl groupings in place of the disulfide linkage in oxytocin. Furthermore, if the proposed structure for oxytocin were correct, reduction of oxytocin with sodium in liquid ammonia followed by addition of benzyl chloride should give rise to the S,S'-dibenzyl derivative of reduced oxytocin, possessing the structure shown in Fig. 4.

Since benzylation of reduced oxytocin had led to inactivation in the earlier study, the expectation was that this benzylated derivative of reduced oxytocin would be biologically inactive. With what we now knew of the structures involved, we could see no reason why treatment of the S,S'-dibenzyl derivative of reduced oxytocin with sodium in liquid ammonia should not lead to the biologically active, reduced oxytocin; oxidation of the sulfhydryl form by aeration should then lead to the regeneration of oxytocin itself, if our concepts and line of reasoning were valid.

We therefore decided to investigate benzylation and debenzylation of reduced oxytocin, starting with highly purified oxytocin preparations in work with Gordon (56). Our best sample of natural oxytocin was treated with sodium in liquid ammonia followed by the addition of benzyl chloride to the liquid ammonia solution. From this reaction mixture, a product was obtained, which on analysis for amino acids had the expected composition, containing 2 moles of S-benzylcysteine along with 1 mole of each of the other seven amino acids present in oxytocin.

This material was biologically inactive. The isolated dibenzyl derivative was then dissolved in liquid ammonia, and metallic sodium was added. After the ammonia had been removed, the product was dissolved in water and oxidized by passing air through the solution at a pHclose to neutral; a biologically active product was obtained. From a comparison of the physical, chemical, and biological properties of the starting and regenerated material, we were convinced that oxytocin had been regenerated from its S,S'-dibenzyl derivative. With this result, I was confident that the door was opened for a synthesis of oxytocin.

If the linear dibenzyl nonapeptide shown in Fig. 5, which possesses a carbobenzoxy group on the amino group of one of the cysteine residues, could be synthesized, it should be possible to convert this protected nonapeptide by reduction to the reduced form of oxytocin and, by subsequent oxidation, to oxytocin (see Fig. 5).

The parallelism between this approach to the synthesis of reduced oxytocin and the approach to the synthesis of glutathione, which I have already discussed, is at once apparent. In both cases, the cysteine residues present in the intermediates for the syntheses were protected by carbobenzoxy and benzyl groups, which were to be removed later in the synthesis by sodium in liquid ammonia.

### Synthesis

After a consideration of the many ways in which the synthesis of the intermediate for oxytocin might be approached, and after exploratory synthetic studies, it was decided to attempt to prepare the compound by combining the tetrapeptide amide, S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, through appropriate means, with L-isoleucyl-L-glutaminyl-L-asparagine to obtain the heptapeptide amide, L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, and to condense the latter with N-carbobenzoxy-S-benzyl-Lcysteinyl-L-tyrosine, thus forming the desired nonapeptide amide. We concentrated on this line of approach. I should like to acknowledge the splendid collaboration of Ressler, Swan, Roberts, and Katsoyannis (53, 57) in this work.

In addition to the classical methods for the formation of peptides, other recently developed procedures were employed. We were able to utilize to particular advantage the contributions of Vaughan and Osato (58) and of Anderson, Blodinger, and Welcher (59). In the former case, the mixed anhydride of an N-substituted amino acid, with, for example, isovaleric acid, is used for the preparation of the peptide; in the latter method, tetraethyl pyrophosphite is the condensing agent.

In addition to using the carbobenzoxy group as a protective grouping for the amino group, we have also employed the tosyl group for this purpose. Removal of the tosyl grouping was accomplished by the use of sodium in liquid ammonia, as utilized in our work with Behrens (60) on the synthesis of  $\alpha$ -amino-N-methyl-Lhistidine. The tosyl group as a protective group in peptide synthesis was first used by Schoenheimer (61), who employed a hydriodic acid-phosphonium iodide cleavage.

Concerning the specific steps in our attempted synthesis of the hormone, the tetrapeptide amide was prepared with Ressler (53, 57, 62). Ethyl carbobenzoxy-L-leucylglycinate was synthesized by the mixed-anhydride procedure of Vaughan and Osato (58). After catalytic removal of the carbobenzoxy group, according to the procedure of Bergmann and Zervas (5), the ethyl L-leucylglycinate was condensed with carbobenzoxy-L-proline, again by use of the mixedanhydride procedure with isovaleryl chloride.

After removal of the carbobenzoxy group by reduction with hydrogen in the

presence of a palladium catalyst, the ethyl L-prolyl-L-leucylglycinate was converted to the tetrapeptide, S-benzyl-Lcysteinyl-L-prolyl-L-leucylglycine, via the dicarbobenzoxy - L - cystinyl derivative. dicarbobenzoxy-L-cystinyl-bis(L-Ethvl prolyl-L-leucylglycinate) was saponified in aqueous dioxane at 5°C in the presence of 1 equivalent of sodium hydroxide to yield the corresponding acid. The latter was converted to S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine according to our procedure for the removal of carbobenzoxy groups in cystine-containing compounds by the use of sodium in liquid ammonia (7) followed by benzylation (6) of the sulfur of the reduced compound in the same medium. The crude tetrapeptide was readily converted, with the use of benzyl alcohol and dry hydrogen chloride, to the crystalline benzyl ester hydrochloride, benzyl S-benzyl-Lcysteinyl-L-prolyl-L-leucylglycinate hydrochloride. Amination was accomplished by allowing the free base obtained from the benzyl ester hydrochloride to stand in solution in methanolic ammonia for several days at room temperature. S-Benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide was isolated from its benzyl ester hydrochloride as a crystalline hydrate.

A novel method was devised with Swan (53, 57, 63) for the synthesis of glutaminylasparagine. Harington and Moggridge (64) had already shown that





heating tosyl-L-glutamic acid under reflux with acetyl chloride gave the mixed anhydride of 1-tosylpyroglutamic acid and acetic acid, which on hydrolysis in aqueous dioxane gave 1-tosylpyroglutamic acid, and that this compound with phosphorus pentachloride gave 1-tosylpyroglutamyl chloride. They had shown that the pyrrolidone ring in these compounds could be opened with aqueous alkali but was stable to anhydrous ammonia. In our work, tosyl-L-glutamic acid was treated directly with phosphorus pentachloride. The reaction product was then coupled with L-asparagine in the presence of aqueous magnesium oxide as the condensing agent (5) to give 1-tosylpyroglutamyl-L-asparagine. The latter compound with concentrated aqueous ammonia yielded tosyl-L-glutaminyl-Lasparagine, the pyrrolidone ring having been opened by addition of the elements of ammonia. Detosylation of the tosyl dipeptide by treatment with sodium in liquid ammonia gave L-glutaminyl-Lasparagine.

For the preparation of tosyl-L-isoleucyl-L-glutaminyl-L-asparagine with Katsoyannis (53, 57, 65), tosyl-L-isoleucine was converted to the corresponding acid chloride and the latter was then coupled with L-glutaminyl-L-asparagine in the presence of magnesium oxide to give the tosyl tripeptide.

Condensation of this tripeptide with the tetrapeptide was effected by the use of tetraethyl pyrophosphite according to the general procedure of Anderson, Blodinger, and Welcher (59). Removal of the tosyl group from the protected heptapeptide amide was effected with sodium in liquid ammonia, by which the benzyl group was also removed. The resulting reduced compound was then benzylated with benzyl chloride in the same medium to yield the heptapeptide amide.

Finally, the heptapeptide amide was condensed with S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosine (66), a dipeptide which had originally been prepared by Harington and Pitt Rivers (67). The condensation was brought about in the presence of tetraethyl pyrophosphite to yield N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide.

This was the compound we had set about to prepare as our proposed intermediate for the synthesis of oxytocin. If our structure for oxytocin were correct, we had every reason to believe that this compound, on reduction with sodium in liquid ammonia, followed by appropriate oxidation, would give us oxytocin (Fig. 5).

Therefore, this intermediate was treated with sodium in liquid ammonia, by which both the benzyl and carbobenzoxy groups were removed. The re-

duced material that was obtained after evaporation of the ammonia was oxidized by aeration in dilute aqueous solution at pH 6.5 and tested for biological activity by the rat uterine strip method (68, 69)and by the chicken vasodepressor method of Coon (70). The latter utilizes the property of oxytocin of lowering the blood pressure of the fowl and has been adopted by the United States Pharmacopeia as the method of assay for oxytosin (40). Based on the assumption that the synthetic material has a potency equivalent to that of the purified natural material (500 units per milligram), the yield of biologically active material, as determined by the two types of assay, ranged in several runs from 20 to 30 percent of the calculated amount from the protected nonapeptide intermediate. The yield approximated closely the yield of oxytocic activity obtainable under similar conditions from the S,S'-dibenzyl derivative of reduced natural oxytocin (56).

#### Synthetic Oxytocin

The purified synthetic product, isolated by countercurrent distribution, and our best samples of natural oxytocin were assaved against each other following rigorously the procedures outlined by the United States Pharmacopeia. The results indicated that the activity of the synthetic material (53, 57) was indeed very close to that of the natural oxytocin (33).

This synthetic material and natural oxytocin were then compared by a battery of physical and chemical tests (53, 57). They had the same amino acid composition. They showed, within experimental error, the same optical activity, partition coefficient (in two different solvent systems), electrophoretic mobility (at two different hydrogen-ion concentrations), infrared pattern, ultraviolet spectrum, and effluent pattern from an IRC-50 resin (71).

The synthetic material formed an active flavianate that had the same crystalline form (fine, silky needles) and the same melting point as the flavianate obtained from natural oxytocin.

On treatment with bromine water, the synthetic material underwent the cleavage into two fragments encountered with natural oxytocin, both giving rise to β-sulfoalanyldibromotyrosine and a sulfonic acid heptapeptide (51).

Sedimentation studies on the molecular weight of natural oxytocin and the synthetic material were kindly made by Schachman and Harrington of the University of California. The natural and synthetic materials behaved identically, and the values that were obtained were in the expected range.

We were fortunate at this stage to have the collaboration of the Lying-In Hospital group of the New York Hospital-Cornell Medical Center on the use of our highly purified natural oxytocin in induction of labor and in milk ejection, for the natural and synthetic material were now compared on human subjects. The synthetic product was fully effective in stimulating labor in the human being and in milk ejection and could not be distinguished from the natural oxytocin in its action. Approximately 1 microgram of either the natural oxytocin or the synthetic material given intravenously to recently parturient women induced milk ejection in 20 to 30 seconds (43).

These comparisons of the physical, chemical, and biological properties of the synthetic product with those of the purified, natural oxytocin justified in our estimation the conclusion that the synthetic octapeptide amide is oxytocin and that the structure shown in Fig. 3 represents that of the hormone (57).

With the establishment of the structure of oxytocin, a new ring system-that is, a 20-membered ring involving a disulfide linkage-was for the first time recognized in nature. Our studies have indicated that a ring of this same size is present in vasopressin, the blood-pressure-raising and antidiuretic hormone of the posterior pituitary (72). Recently it has been shown by Sanger and coworkers (73) that a ring of the same size, involving a disulfide linkage, occurs in the insulin molecule, as part of a more involved structure.

The establishment of the structure of oxytocin and vasopressin will undoubtedly open the door to a better understanding of these hormones by the biochemist, the physiologist, the pharmacologist, and the clinician. Moreover, it should provide a suitable basis for the study of the relationship of chemical structure to biological activity in these proteinlike substances. The synthesis of oxytocin will afford a means of obtaining the compounds necessary to the study of this relationship and may, in addition, point the way to the synthesis of more complex sulfur-containing polypeptides.

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# Charles C. Adams, Ecologist

Charles Christopher Adams was a pioneer in and one of the creators of ecological perspective. Through the kindness of his daughter, Harriet Adams, I have at hand his bibliography of some 154 titles, including six unpublished works. This list makes clear not only the gradual development of his personal interests, but also his service in broadening our concepts of ecology and its role. Although it is not a matter of record, much that he accomplished was done in the face of inertia and even opposition on the part of established and conventional influences.

Adams was born in 1873. He began publishing notes on natural history before his graduation from Illinois Weslevan University in 1895. Several papers on invertebrates appeared after he did his master's work at Harvard University in 1899, but it was his fellowship at the University of Chicago (1900-1903) that appears to have been decisive. Here his contacts with Cowles, Salisbury, and Transeau resulted in notable papers on base leveling and on Pleistocene climatic change in relation to faunal problems. Together with Transeau's paper on forest centers, these marked a resumption of serious American biogeographical study, initiated long before by Asa Gray, but with the added advantage of new information on geomorphology and Pleistocene history.

From 1903 to 1907, Adams was in museum work, first at the University of Michigan, then at the University of Cincinnati. He quickly developed the idea that the museum is a vital teaching center that is linked to the community and its natural history. This idea was to remain a guiding motive for the rest of his life;

it expressed itself at this time in his ecological survey of central and northern Michigan. In 1904 he organized and directed the Isle Royale Ecological Survey. In 1908 he returned to Illinois as associate in animal ecology, where he prepared his Guide to the Study of Animal Ecology and a detailed report on the various communities of invertebrates in the prairies and forests of eastern Illinois.

In 1914 he became forest zoologist of the New York State College of Forestry at Syracuse. There he organized the Roosevelt Wildlife Experiment Station and directed its activities until he became director of the New York State Museum, a position he kept until his retirement in 1943. At these two posts, much of his most significant work was done, although it was often concealed behind the routine into which it was incorporated. He continued his idea of ecological surveys at the Palisades, Mount Marcy, Alleghany State Park, and in the Rochester region. He wrote much on wildlife and fisheries in relation to forestry, and after an initial paper on conservation in 1915, he gave that problem increasing emphasis.

Ecologists, notably in the Forest Service, had long appreciated the possibilities of their subject as an applied science. But it seems to me that Adams did more than any other individual in America to give this idea comprehensive form. In 1930 he turned his attention to the synthesis with human ecology, using the Shaker collection in the Albany Museum as a basis. In orderly fashion, he proceeded to enlarge the scope of his interest, through schools, water resources planning, and regional and urban planning.

He brought to the attention of ecologists the work of Mumford, Mackaye, Benedict, and Patrick Lindeman. Geddes, whose concepts of the living museum and of ecological planning and whose experience, in some respects, paralleled his own. Adams organized symposia on human ecology and insisted that ecology, with its tremendous possibilities for human good, ought to be underwritten on the same scale as geography, geology, and the physical sciences.

Keenly aware that ecology is a study of process, he saw the importance to it of records and archives, and of some central institute where these could be conserved and where workers could be housed with facilities fitting their value to society. He never ceased trying to rouse ecologists to appreciate their own importance, warning them that unless they could do so, their job would be taken over eventually by others.

One of the founders and a past president of the Ecological Society of America, he was also active in the Association of American Geographers. He maintained an extensive and lively correspondence, and he was unusually enterprising in hunting up those whose ideas interested him. Physically he was stout and vigorous. His tastes were simple, but his energy prodigious. His home was itself a museum and library combined, overflowing with books and pamphlets which he governed by his own mysterious kind of order.

At the time of his death in 1955, he was working on a Guide to the Study of Human Ecology: The Dynamics and Processes of Orientation and Integration. Like his earlier guide to animal ecology, this was to be an annotated bibliography, but so set up as to be immensely useful to any serious student.

His library, notes, and other material have been presented by his daughter to Western Michigan State College at Kalamazoo, where they will be kept available, as he wished that all important ecological materials might be, for future workers. There they will form a nucleus for the Charles C. Adams Center for Ecological Studies under the direction of Daniel Jackson.

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