

V I N C E N T   D U   V I G N E A U D

## A trail of sulfa research: from insulin to oxytocin

*Nobel Lecture, December 12, 1953*

It is a pleasure to have the opportunity of discussing with this Academy some of the researches that I have been interested in during the past thirty years. I am deeply grateful to the Academy for the recognition, as expressed in the terms of the citation, of our work on "biochemically important sulfur compounds". You have also noted in the citation "the first synthesis of a polypeptide hormone". This hormone, oxytocin, the principal uterine-contracting and milk-ejecting hormone of the posterior pituitary gland, is a sulfur-containing compound, and its synthesis was the culmination of many experiences along a trail of research stemming from my original interest in sulfur and in insulin.

It would seem proper, therefore, with the citation in mind, that I should discuss with you a "trail of sulfur research". I shall try to unravel some of the stepwise evolvment of our researches from insulin to oxytocin. I shall also try to bring out 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 this trail I am going to present could not have been worked out.

I have also been fortunate in the kind of support that I have received from various foundations, such as the Rockefeller Foundation; from industrial firms, such as Parke, Davis and Company, Eli Lilly and Company, and the Lederle Laboratories Division of the American Cyanamid Company; from a governmental agency, namely the National Institutes of Health; and from the universities where I have had the pleasure of working. All of this support was unrestricted, allowing us to explore whatever trail we wished.

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 towards 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 breaking through the confines of the known world.

As one looks back on a trail of research, the continuity is sometimes greater than one 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 sulfur-containing compounds, such as cystine, homocystine, and methionine, and to study their metabolic significance in transsulfuration and transmethylation. These researches diverted us into interesting by-paths. 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 which we gained along the route, were vital when we came to study the posterior pituitary hormones.

Now where did the sulfur trail start?

I think it started at the University of Illinois, where my first teacher in biochemistry was the late Professor 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 Professor 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 Professor Marvel was concerned with the synthesis of compounds which we hoped would have a local anaesthetic effect accompanied by an epinephrine-like 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 Professor 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 will recall the thrill of

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

While there, I became intrigued with the fact that all of our preparations contained sulfur, and most of my efforts over the next two 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<sup>1</sup>.

The following year, while working in Professor 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 the cystine in pure form. This isolation was eventually accomplished<sup>2</sup>. As I continued work at Hopkins on insulin in collaboration with Jensen and Wintersteiner, we could find nothing but ordinary amino acids and ammonia in acid hydrolysates of insulins<sup>3,4</sup>.

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 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.

Although the peptides we eventually made did not have hypoglycemic activity, the work thereon 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 reac-

tions involving sodium and liquid ammonia, which, almost twenty years later, played a vital role in our synthesis of oxytocin, which will be discussed subsequently. 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 synthetic steps 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 groups. 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 very convenient method for the synthesis of cystine peptides might result. In work with Audrieth and Loring<sup>6</sup>, we had already prepared cysteine from cystine by this method of reduction.

The reduction of dicarbobenzoxycystine was therefore attempted in work with Sifferd<sup>7</sup>. 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 cysteine in liquid ammonia with metallic sodium, cystine being isolated after oxidation<sup>7</sup>.

This same reductive procedure was also applied to the preparation of cystinylbisglycine from dicarbobenzoxycystinylbisglycine and of cysteinylglycine from *S*-benzylcysteinylglycine<sup>8</sup>.

The effectiveness of these reactions impressed us with their potentialities as possible key reactions for a synthesis of glutathione, the structure of which

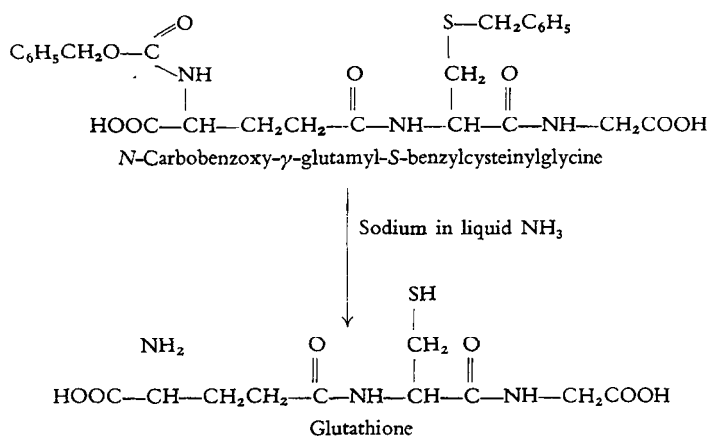


Fig.1. A synthesis of glutathione.

was believed, through the work of Hopkins<sup>9</sup> and of Kendall, Mason, and McKenzie<sup>10</sup> to be  $\gamma$ -L-glutamyl-L-cysteinylglycine. If we could synthesize *N*-carbobenzoxy- $\gamma$ -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<sup>11</sup>, we were able to obtain this desired intermediate by the coupling of suitable derivatives of the three amino acids involved. As 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<sup>12</sup>.

While this synthetic work was going on, we continued our work on insulin, along mainly two lines. One was concerned with 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 seven years of work, we finally were able to account for the sulfur of insulin entirely on the basis of cystine<sup>13</sup>, and we could obtain no evidence for any sulfur compound other than cystine in insulin<sup>14,15</sup>.

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<sup>16-18</sup>. Reoxidation did not restore activity.

This work on insulin aroused our interest in other protein or protein-like 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 polypeptide-like 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 per cent cystine<sup>19</sup>. Of course we couldn't tell at that time whether the cystine was present in the hormone or in the impurities. Nevertheless, in work with Sealock<sup>20</sup>, 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, then 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 non-reduced 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<sup>20</sup>.

We also investigated the behavior of the vasopressin preparation upon 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.

The purification was a slow process, as the amount of active principles in the gland is extremely small, they 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<sup>21-26</sup>.

We laid aside the problem during the war period for certain assignments, particularly on penicillin, but thereafter the isolation of oxytocin was undertaken in collaboration with Livermore<sup>27</sup>. Since the countercurrent distribution technique developed by Craig<sup>28</sup> 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 co-workers<sup>29</sup>. The source material for preparation of the oxytocin fractions was a commercial extract provided by Dr. Kamm of Parke, Davis and Company. The countercurrent distribution between 0.05 per cent 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<sup>30</sup> we were able, with Pierce<sup>31</sup>, to show that an acid hydrolysate of oxytocin consisted of eight amino acids and ammonia.

It was then 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<sup>32</sup>. A preparation was obtained which 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 is shown in Fig. 2. The amino acids were present in a molar ratio to each other of 1:1, and the molar ratio of ammonia to any one amino acid was 3:1. Molecular weight determinations indicated a molecular weight in the neighborhood of 1,000.

The sulfur content of oxytocin could be entirely accounted for by cystine.

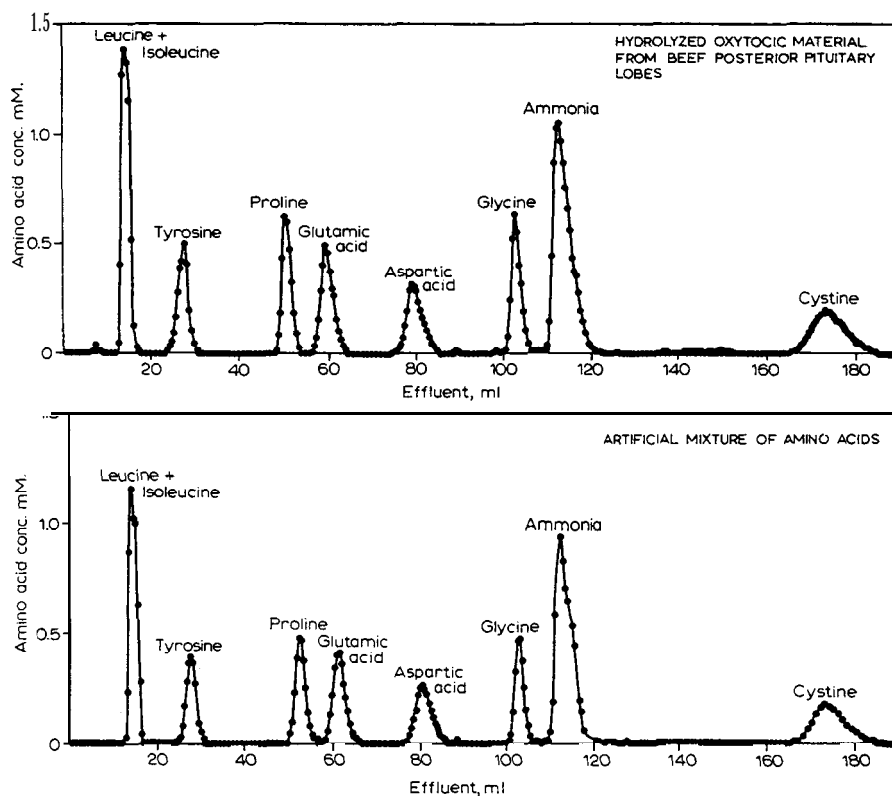


Fig. 2. Separation of amino acids from a hydrolysate of oxytocin (*upper chromatogram*) and from an artificial mixture of amino acids simulating the composition of oxytocin (*lower chromatogram*). Solvents, 1:2: 1 *n*-butyl alcohol - *n*-propyl alcohol - 0.1 *NHCl* followed by 2:1 *n*-propyl alcohol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

Knowing the cystine content of the purified hormone, it becomes evident, from the sulfur content of all of the pre-war preparations including those of Kamm and co-workers and our preparations, that none of these preparations could have been more than 50 per cent 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 1,000 transfers resulted in no change in composition, and this work<sup>33</sup> 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 which had a distribution curve approximately the same as that from the beef glands<sup>3,3</sup>. 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.

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 sixty years ago that the first biological effect of the pituitary gland was discovered by Oliver and Schäfer<sup>34</sup>. 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<sup>3,5</sup>. Since that time, other biological activities of posterior pituitary extracts were noted, particularly the uterine-contracting, or oxytocic, effect by Dale<sup>36</sup> in 1906; the milk-ejecting effect by Ott and Scott<sup>37</sup> in 1910; the blood-pressure-lowering effect in birds, the so-called avian depressor effect, by Paton and Watson<sup>38</sup> in 1912; and the inhibition of urine excretion in man, the antidiuretic effect, by Von den Velden<sup>39</sup> in 1913.

As to 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 U.S. Pharmacopoeia<sup>40</sup> and found to possess this activity<sup>33</sup> to the extent of 450 to 500 units per mg. 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 uterus - the uterine-contracting activity. The oxytocin also showed the same potency (450 to 500 units per mg, 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<sup>41</sup>, in rabbits by Cross and Van Dyke<sup>32</sup>, and in recently parturient women, the

latter testing having been carried out in a collaborative study with Douglas, Nickerson, and Bonsnes of our Department of Obstetrics and Gynecology<sup>43</sup>.

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 mg. 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<sup>44</sup>. It might be mentioned that vasopressin, in addition to its pressor and antidiuretic effects (500 to 600 units per mg, relative to the standard powder), also possesses avian depressor, uterine-contracting, and milk-ejecting activity, but the potency of vasopressin with respect to the latter three activities is only a fraction of the potency of oxytocin.

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 performed 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<sup>45-50</sup>, and the final decisive experiments with Ressler and Trippett<sup>51,52</sup> 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<sup>52,53</sup> of the structure of oxytocin, a new type of cyclic polypeptide amide shown in Fig. 3.

Although this structure was the only one that we could arrive at through the rationalization of our data, we felt that synthetic proof of this structure 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<sup>54</sup>. Tuppy's proposal was based on the data from our labora-



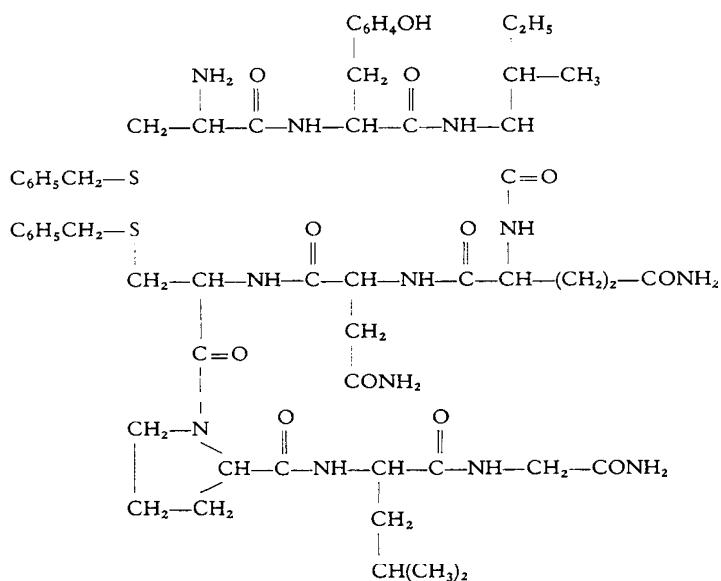


Fig. 4. Benzylated derivative of reduced oxytocin.

ture 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 the benzylation and debenzylation using highly purified oxytocin preparations in work with Gordon<sup>56</sup>. 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.











and co-workers<sup>73</sup> 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 protein-like 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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