I find it intriguing to contemplate how one starts out on a trail of ex-

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[1].

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[2]. 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[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

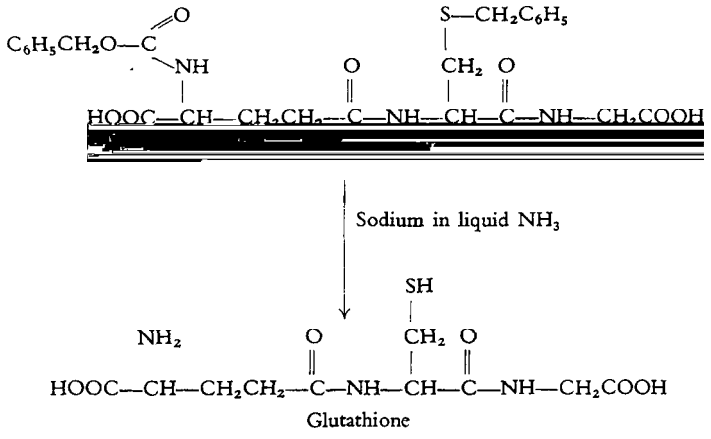


Fig.1. A synthesis of glutathione.

was believed, through the work of Hopkins[9] and of Kendall, Mason, and McKenzie[10] to be γ-L-glutamyl-L-cysteinylglycine. If we could synthesize *N*-carbobenzoxy-γ-glutamyl-S-benzylcysteinylglycine, we felt that its re-

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.

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[19]. 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[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, 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[20].

We also investigated the behavior of the vasopressin preparation upon

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[21-26].

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[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 co-workers[29]. 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[30] we were able, with Pierce[31], 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[32]. 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.

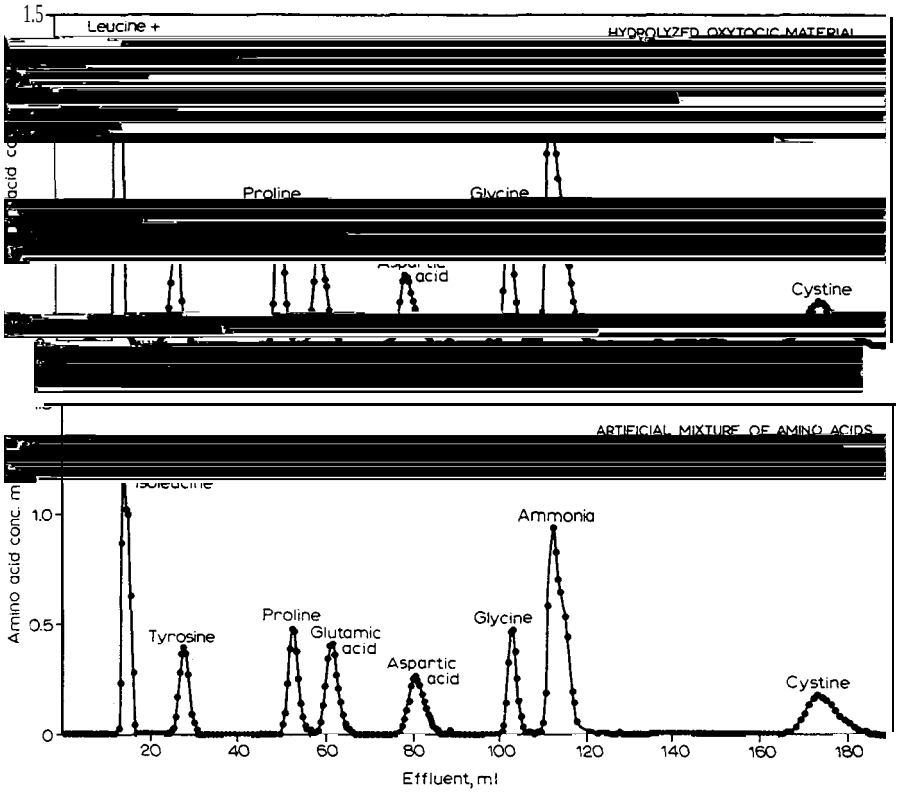


Fig. 2.Separation of amino acids from a hydrolysate of oxytocin *(upper chromatogram)* and from an artificial mixture of amino acids simulating the composition of oxytocin *(lower chromatogram).* Solvents, *1:2:* 1 *n*- butyl alcohol - *n*- propyl alcohol - 0.1 *N* HCl followed by 2:1 *n*- propyl alcohol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

Knowing the cystine content of the purified hormone, it becomes evident, from the sulfur01.2 Tm (Kno 240 287.e88.72 a1 >>8 BT 0ugre6e588 C /4.msa148 Tm (uch-U9

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[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.

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[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[36] in 1906; the milk-ejecting effect by Ott and Scott[37] in 1910; the blood-pressure-lowering effect in birds, the so-called avian depressor effect, by Paton and Watson[38] in 1912; and the inhibition of urine excretion in man, the antidiuretic effect, by Von den Velden[39] 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[40] and found to possess this activity[33] 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[41], in rabbits by Cross and Van Dyke[32], 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[43].

We thought at first that oxytocin was devoid of pressor and antidiuretic

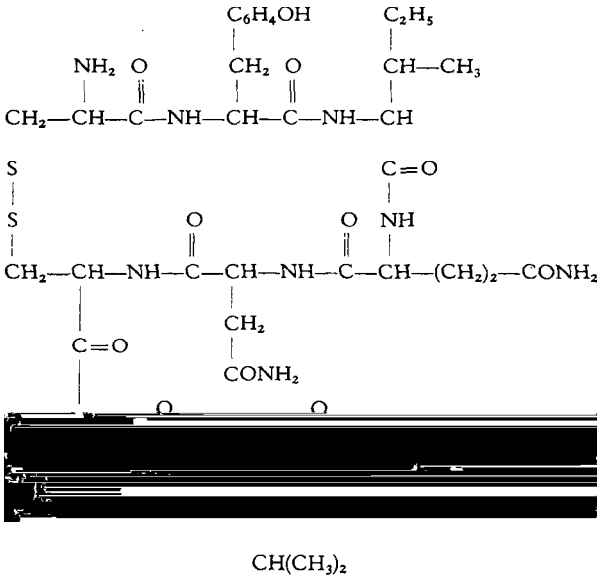
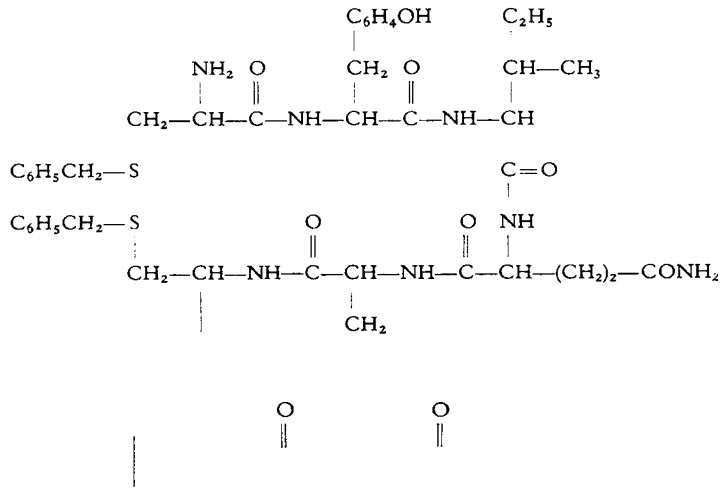


Fig. 3. Oxytocin.

tory 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 which 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 already mentioned, we felt that synthetic proof of the structure we had arrived at had to be adduced; 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 groups 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.

The clue to the synthetic approach to 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 struc-



From this reaction mixture, a product was obtained, which on amino acid analysis had the expected composition, containing two moles of *S*-benzylcysteine along with one mole of each of the other seven amino acids present in oxytocin.

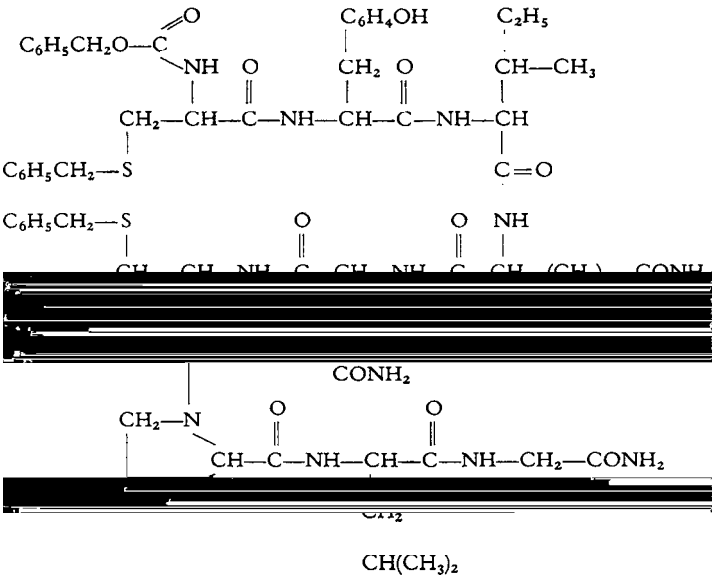
This material was biologically inactive. The isolated dibenzyl derivative was then dissolved in liquid ammonia and metallic sodium was added. After removal of the ammonia, the product was dissolved in water and oxidized by passing air through the solution at a pH close to neutral; a biologically active 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 to a synthetic attack on oxytocin.

If the linear dibenzyl nonapeptide shown in Fig. 5, which possesses a carbobenzoxy group on the ammo 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 residue(s) present in the intermediates for the syntheses were protected by carbobenzoxy and benzyl groups, which were to be removed in the last synthetic step by sodium in liquid ammonia.

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-L-cysteinyl-L-tyrosine, thus forming the desired nonapeptide amide, and we concentrated on this line of approach. In this work I should like to acknowledge the splendid collaboration of Ressler, Swan, Roberts, and Katsoyannis[53,57].

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 An-



Protected nonapeptide intermediate

according to the procedure of Bergmann and Zervas[5], the ethyl-L-leucylglycinate was condensed with carbobenzoxy-L-proline, again by use of the mixed anhydride procedure with isovaleryl chloride.

After removal of the carbobenzoxy group by reduction with hydrogen in the presence of palladium catalyst, the ethyl L-prolyl-L-leucylglycinate was converted to the tetrapeptide, *S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine via the dicarbobenzoxy-L-cystinyl derivative. Ethyl dicarbobenzoxy-L-cystinyl-bis(

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

lowing rigorously the procedures outlined by the U. S. Pharmacopoeia. The results indicated that the activity of the synthetic material

and co-workers[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. More-

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