A Trail of Research

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IN

Sulfur Chemistry and Metabolism

AND RELATED FIELDS

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THIS account of a "trail of research" is dedicated to the loyal and enthusiastic group of associates who have traveled this trail with me.

Preface

IN THE Messenger Lectures of 1950, which form the substance of this small volume, an attempt was made to retrace the research trails originating from a study of insulin that I have had the pleasure of working out in association with various collaborators over a period of twenty-five years. I attempted to present not only the findings encountered, but also in many instances the stepwise evolvement of these findings, including the accidents of fate that played a part. I wished to show how one trail led to another, and to bring out the thinking behind the decisions which were made in the following of one trail and then another.

One may attempt to work out a trail of research from the work of others as gleaned from the formal literature of a subject; but in reality it is not possible to do so without a great deal of rationalization, because the kind of facts that are necessary for this understanding seldom appear in scientific papers.

It is intriguing 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. True exploratory research is really the working out of a winding trail into the unknown. The investigator who is attracted to this type of research is attracted by the same thrill, albeit at a sublimated level, that was once enjoyed by the explorers in breaking through the confines of the old world. This is the kind of research that our academic and governmental administrations must be very careful to preserve.

They must encourage the desire and guarantee the freedom for such exploration into the unknown, just as it was necessary and profitable to the administrations of the old world to allow individuals to explore the unknown geographic world.

Project research is, of course, necessary. There are certain needs of society that must be met as soon as possible for its immediate benefit; but it is also necessary to preserve this other type of exploratory research. The latter must be guarded from attenuation by those who do not understand that this type of research is vital for our future.

I have had the privilege and the thrill of following this kind of research through twenty-five years. I have been accompanied in the various stages of this exploration by a group of fine and loyal associates. I have also been fortunate in the kind of support that I have received from various foundations and industrial firms, and from the universities where I have had the pleasure of working. No "strings" have ever been tied to any support that I have received, and for this I am truly grateful.

The only occasion we had to turn away from the trails of our own choosing was during World War II, when it was necessary to hold in abeyance exploration which we had under way. We were asked to explore other territory. But even then the two assignments were for exploration at the fundamental level: the one on vesicants and the other on penicillin. This allowed us to make certain contributions that we could not otherwise have made, such as the contribution to the synthesis of penicillin.

It has so happened, curiously enough, that nearly all our researches have been built around sulfur in some way or another. However, the fact that this is so has not been entirely an accident. All of our researches which were presented in the Messenger Lectures can be traced back step by step to a single main trail—to a study of insulin, a study which we embarked upon some twenty-five years ago. As it turned out, insulin was found to be a sulfur-containing compound, and it was the sulfur that particularly

attracted our attention; from here the various trails branched forth and in turn branched again.

There is, however, one study involving a sulfur compound upon which we spent a great deal of time in our laboratory, but which did not evolve from this stepwise trail, branching out from the chemistry of insulin. When we undertook this investigation, this substance was thought to be sulfur-free. I shall not discuss this field of research in the ensuing chapters since it was not a direct branch of the trail under discussion. It may, however, be amusing to point out how this particular compound became a part of our researches and became identified as a sulfur compound. The problem I refer to was the one on vitamin H, which we identified as biotin in collaboration with Dr. Paul György and Dr. Donald Melville, and the chemical structure of which we worked out mainly with Dr. Melville and Dr. Klaus Hofmann. While I was in Washington at the George Washington University Medical School, Dr. György came to my laboratory in 1937 and asked if I would collaborate with him on a problem having to do with vitamin H, the anti-egg-white-injury factor. It had already been reported in the literature that vitamin H was a sulfur-free substance and it was suspected that vitamin H might be some kind of an amino acid. I accepted György's invitation because we had been much intrigued through the years by the remarkable effects of this unknown compound in counteracting egg-white injury. As an amino acid problem it appealed to me as a project for our group. During the next few years of exploration of this problem we were able to show that this mammalian vitamin-like factor was identical with the yeast-growth-promoting factor biotin, a sulfur-containing compound isolated some years before by Kögl. That vitamin H turned out to be a sulfur compound was indeed a surprise to us.

I have mentioned, in fact I have emphasized, that the chemistry of insulin and of sulfur has formed the background of much of our research endeavors. Why, I wonder, should this be true? There is a certain amount of accident involved, but the background of interest

and background of training, I think, did play a role. I would trace it back to two individuals who influenced my development as an undergraduate, Professor H. B. Lewis, with whom I had my first course in biochemistry at the University of Illinois in 1921, and Professor W. C. Rose, with whom I had an advanced course in biochemistry in 1923. Can it be an accident when one develops an interest in the chemistry and metabolism of sulfur compounds if one has had such an enthusiastic and inspirational teacher as Lewis in one's first course in biochemistry? His enthusiasm for sulfur was unbounded. Those who have heard Professor Rose lecture know what an inspirational teacher he is. I well recall listening to a lecture he gave in 1923 after his return from Toronto in which he enthusiastically discussed the exciting discovery of Drs. Banting and Best on insulin. I recall the thrill of listening to him and the curiosity that was aroused in me as to the chemical nature of this compound that could bring about the miracles he described.

In a certain sense the trail of research that formed the basis of the Messenger Lectures started with this lecture of Professor Rose. From the actual experimental standpoint the trail of research started in the laboratory of the Philadelphia General Hospital two years later in 1925 in a metabolic study undertaken with Dr. Walter G. Karr. This work was concerned with the effect of fasting on glucose tolerance in the rabbit. When the marked effect of starvation on decreasing the tolerance for glucose was established, the effect of diet and of certain other substances on glucose tolerance was investigated. With an interest aroused in insulin as already explained, it was natural for us to want to know the effect of insulin. Although insulin of course increased the tolerance for sugar when administered simultaneously with the sugar, the striking observation was made that the administration of insulin a few hours before the administration of the test dose of sugar greatly decreased the sugar tolerance. Thus having observed firsthand the dramatic effect of insulin on the organism, I could not help but become intrigued as a chemist as to what manner of compound could produce such effects.

While engaged in this study I received an invitation from Professor John R. Murlin at the University of Rochester Medical School, which had just opened its doors, to come and work on the chemistry of insulin in his Department of Vital Economics, a department devoted mainly to endocrinology and metabolism. The chance to work on the chemistry of insulin transcended all other interests, and I accepted Professor Murlin's invitation. It may be recalled that Professor Murlin had had an intensive program under way on this pancreatic hormone for several years and, in fact, had independently prepared active extracts at the time of the announcement by Banting and Best. His active program on insulin had been continued, and it was therefore a rare opportunity to one of chemical leanings to enter a laboratory with such an investigator as Professor Murlin with his background of experience with this hormone.

With these few comments on the nature and importance of fundamental research and the brief discussion of the background which tends to influence the research trails one follows, I would like to present the actual trail of researches originating from our earlier studies on the chemistry of insulin.

Contents

I

From Insulin to Homocystine

EARLY in the work on insulin it was suspected by various workers that insulin might be a sulfur-containing compound. This suspicion grew into a definite possibility as the result of the work of Professor Abel and his associates at the Johns Hopkins University Medical School, who later crystallized insulin for the first time (1-3). Abel and Geiling (1) found that the amount of sulfur easily split out by weak alkali paralleled the activity of certain amorphous preparations and that, when the sulfur was split out, the activity was destroyed. These findings led them to ask "to what extent, if any, the Islets of Langerhans are dependent upon the presence in our food of a special labile sulfur compound, a precursor indispensable for the elaboration of the hormone, in the absence of an adequate supply of which, pathological alterations in the cells of the Islets of Langerhans would take place." It was quite clear that the question of the identity of the sulfur moiety had become one of paramount importance.

At the time of this announcement by Professor Abel we were obtaining evidence that insulin contained cystine. The labile sulfur could be accounted for on the basis of the presence of cystine in peptide linkages. The sulfur of cystine was quite stable to weak alkali, which would split out the sulfur of insulin. However, as pointed out by Brand and Sandberg (4), the sulfur of cystine peptides such as dialanylcystine was quite labile to weak alkali, and the high degree of lability of insulin sulfur did not necessarily indicate that the sulfur was not cystine sulfur. It was found that, when the labile sulfur was split out of the insulin by treatment with

alkali, the test for cystine was greatly reduced in intensity, which indicated that the cystine and not a strange sulfur compound was the source of the alkali-labile sulfur (5, 6). Furthermore, it was found that, although the sulfur of insulin was labile, the sulfur in the hydrolysate of the insulin became stable like that of free cystine. This change in lability upon hydrolysis was identical with what would be expected of peptides of cystine.

In this work at Rochester we were also able to confirm Abel's crystallization of insulin (2) and found that the crystalline insulin possessed the behavior just discussed. From our studies we came to the conclusion that the sulfur was present as the disulfide linkage and that insulin was most likely a derivative of cystine, and we suggested that the cystine in insulin was linked to the rest of the molecule by peptide linkages (5).

The conclusive demonstration of the actual presence of cystine in crystalline insulin, however, had to rest on isolation; so later, when we had the opportunity of working in Professor Abel's laboratory, we undertook the isolation of cystine from crystalline insulin. Of course, this work had to be carried out on a very small scale, and difficulties were encountered with the isolation of the cystine. We soon recognized that one of the difficulties was that we had partially racemized the cystine during the acid hydrolysis of the insulin, and on looking into the literature we found that racemized cystine was far more soluble than L-cystine. Here our interest in the isomers of cystine began. We found that, in spite of the great amount of work that had been done on the problem, the isomers had not been isolated and that, in fact, there was much difference of opinion as to whether the inactive material was the meso or racemic form or a mixture of the two.

Somewhat later at the University of Illinois, when the opportunity arose, the resolution of cystine was undertaken with Hollander (7, 8) and the fractionation of inactive cystine into the racemic and meso forms with Loring (9, 10). Thus all four isomers in pure crystalline form became available for biological and physical chemical studies. With the availability of these isomers,

our interest was aroused in their utilization by the animal body, and studies were undertaken of their ability to promote growth of the white rat on a cystine-deficient diet. In collaboration with Dorfman and Loring the finding was made that the D isomer could not be utilized for this purpose (11). The oxidation of these isomers (12) and the behavior of the optical isomers of other amino acids in the animal body were then studied. With some of these amino acids such as tryptophan the D form was found to be utilized (13).

Although we found that both D- and L-tryptophan could be utilized for growth purposes, the acetyl-D in contrast to the acetyl-L could not be utilized. This awakened in us an interest in acetyl amino acids. It was also found in this work, done in collaboration with Sealock, that the sodium salt of acetyl-L-tryptophan in aqueous solution at 35 to 40° was completely racemized by acetic anhydride within a few hours (14). This was extended to a study with Meyer of the racemization of other amino acids (15, 16).

The work on tryptophan prompted us later at George Washington University to explore with Irish (17) the conversion of the D isomer in the body to the L. This in turn led to a long trail of research on the significance of the metabolic process of acetylation (18-23). Our researches on acetylation reopened a field of metabolism which was dormant for many years but which is now being actively studied in many laboratories.

To go back now to the isolation of cystine from crystalline insulin—this was readily accomplished when care was taken to avoid, as much as possible, the racemization of cystine and when we had worked out a new procedure that would separate tyrosine from cystine in small quantities. The actual isolation and identification of cystine from insulin demonstrated beyond any argument that cystine was present in the insulin molecule.

In this investigation during 1927-1928 at the Johns Hopkins Medical School, we collaborated with Wintersteiner and Jensen. This work led not only to the isolation of cystine and tyrosine

from crystalline insulin (24) but also to the isolation of histidine, leucine, lysine, and arginine (25) and to a study of the nitrogen distribution of insulin by the Van Slyke procedure (26). This collaborative research established insulin as a protein.

It may seem strange to speak of work establishing insulin as a protein because it is now a generally accepted fact that a hormone can be a protein or that a protein can be a hormone. Yet at that time there was great reluctance in accepting this viewpoint. I probably need not point out that there was a similar battle going on in the field of enzymes as to whether an enzyme could be a protein or a protein could be an enzyme. The old concept that a protein was a type of static building block has now been superseded, and the dynamic role of proteins in the metabolism of the body is one of the keystones of modern biochemistry.

It is a little startling to think that the amino acids when put together in a certain way, in a particular architecture, can lead to such an array of compounds exhibiting such a variety of physiological and pharmacological phenomena. In our early work on insulin we could find nothing in insulin other than ordinary amino acids. We early came to the conception that the architecture of this molecule as a whole was the important factor in the chemistry of insulin. We have had no reason to change this belief since.

This interest in the architecture of combinations of amino acids in relation to pharmacological or physiological activity has been in the background of some of our other explorations. We became interested in investigating certain combinations of amino acids which produced substances of new biological activity. This is one of the reasons why glutathione and carnosine have had a strong appeal to us, since they offered simple examples of this phenomenon. L-Carnosine, composed of β-alanine and L-histidine in peptide linkage, as shown in Fig. 1, is capable of reducing blood pressure, even though neither of the free amino acids has an effect on blood pressure. We became interested in the relation of the organic structure of certain peptides of histidine to their biological activity. We followed this trail over a period of years

with the synthesis of D-carnosine and a variety of analogues and homologues. I should like to describe briefly some of the salient points encountered along this trail.

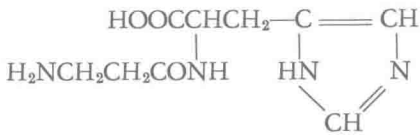

Fig. 1. Carnosine.

D-Carnosine was synthesized in collaboration with Hunt (27) by the method we had originally worked out with Sifferd (28) for L-carnosine. When the D-carnosine was injected into an anesthetized cat in even 20 times the dose of L-carnosine, no depressor action was detected (27). Thus the remarkable specificity of the depressor activity of L-carnosine with respect to spatial configuration was realized.

To study further the relation of structure to biological activity, the α-alanyl peptides of L-histidine, D-alanyl-L-histidine and L-alanyl-L-histidine, were next synthesized, in collaboration with Hunt (29). In the synthesis of these two peptides we encountered the great instability of carbobenzoxy-α-alanyl chloride and noted that the latter was readily converted to the carbonic anhydride. We then found that the carbonic anhydride of D-α-alanine could be condensed with L-histidine methyl ester and that a good yield of the desired peptide could be obtained. The product was identical with that which we had synthesized from the carbobenzoxy-α-alanyl chloride. We pointed out in the presentation of this work that the results afforded a possible method for snythesizing peptides of amino acids where the acid chloride of the carbobenzoxy derivative might be very unstable.

When the effect of L-α-alanyl-L-histidine and D-α-alanyl-L-histidine on the blood pressure of the cat was tested, no appreciable depressor activity was found with either isomer. This result demonstrated the importance of the position of the amino group in the alanyl moiety of carnosine for the production of its