

RECENT PROGRESS IN HORMONE RESEARCH

*Proceedings of the
1970 Laurentian Hormone Conference*

Edited by
E. B. ASTWOOD

VOLUME 27

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1971



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Recent Progress in
HORMONE RESEARCH

The Proceedings of the Laurentian Hormone Conference

VOLUME 27

PREFACE

The speakers on the program of the 1970 Laurentian Hormone Conference set a new standard of excellence, and the favorable comments from the membership gladdened the hearts of the Committee on Arrangements. One innovation contributing to this performance was the introduction by the first speaker, Dr. Blundell, of the use of two projectors for slides and two screens. This added greatly to the clarity of exposition in his difficult task of depicting the tertiary structure of insulin—the overall effect was most pleasing to the audience. This manner of presenting his material was Dr. Blundell's third choice; he would have preferred to use fully stereoscopic projection or, failing this, three screens, but suitable equipment was not available. Other speakers were quick to recognize how the new double projection could be used to advantage in their presentations and promptly reshuffled their slides accordingly.

The article on growth in mammals induced by a tapeworm was enhanced by a presentation shared by a classical helminthologist and an endocrinologist, and this glimpse at comparative physiology was extended by the paper dealing with diabetes in a variety of animals including some exotic ones. And so it went, with novelties and new scientific breakthroughs interspersed throughout the week.

The meeting was held as usual at Mont Tremblant Lodge, Mont Tremblant, Quebec, Canada, August 29 to September 4. Still further improvements in the amenities of the Lodge and the delicious food have subdued the voices of those of the membership favoring moving the meeting about from place to place.

Any favorable comments that might be made about the presentations could equally well be applied to the manuscripts submitted for this volume. Most of them were turned in promptly and all of them were in finished, impeccable order—an editor could not ask for more. The particular interests of the topics covered this year should make this a popular volume. It will be possible for the interested reader to find in one place the most recent work on the glycoprotein hormones and on their unique bimolecular makeup. The extensive reports on the radioimmunoassays of steroid and protein hormones and on the binding of steroid hormones to target tissues will also be of general interest.

The fascinating story growing out of recent work on vitamin D, which makes it seem more like a hormone than an accessory food factor, is told in a reserved but graceful manner by the discoverer of much of the new information. If any one compound were to be singled out as the parent of the steroid hormones, the choice would be pregnenolone, and the authoritative account

of the biosynthesis of this important substance will be a valuable source of reference.

The mediation of the action of many hormones through activation of the enzyme adenylyl cyclase is a topic of wide popular interest at present. The two scholarly papers, one by Dr. Pastan and one by Dr. Garren and their respective collaborators, provide the basis for careful thought and deliberation on the part of those who would understand and evaluate the vast literature in this area.

The last four chapters in this volume derive from a symposium on *in vitro* methods for studies in endocrinology that was held on the last day of the conference. Each speaker was allowed only 20 minutes for his presentation; however, the published material is much fuller and more complete.

The Committee on Arrangements wishes to express its gratitude to the management of the Lodge for their splendid cooperation during the conference and to the members of the staff of Academic Press for the efficient and skillful manner in which they have brought out this hopefully faultless and certainly handsome volume. The Committee is also most grateful to its executive secretary Miss Joanne Sanford and to her helpers Miss Lucy Passalapi and Mrs. Mina Rano for their diligence and skill in making most of the arrangements and for recording and collating the discussion. We also wish to thank the members who served as Chairmen of the sessions, Drs. W. H. Daughaday, A. E. Wilhelmi, D. H. Solomon, S. Lieberman, L. T. Samuels, R. W. Butcher, G. Nichols, and K. Savard.

If the spirit and interest of the last few meetings and the welcome acceptance of the last few volumes can be used as a guide, we can look to the future and confidently anticipate that there will be many more good conferences and a continued demand for annual additions to *Recent Progress in Hormone Research*.

E. B. ASTWOOD

Boston, Massachusetts
May, 1971

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X-Ray Analysis and the Structure of Insulin¹

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I. Introduction

Insulin is one of the most widely studied of all hormone molecules. It is not only central to research on diabetes. It is also one of the smallest of those complex molecules, the proteins, and, therefore, has been the subject of many studies by organic and physical chemists. An important theme in the work of both biologists and chemists has been the effort to understand its three-dimensional structure, and the literature contains many speculative models. Now we are pleased to be able to describe the first detailed results on the structure of insulin—indeed of any protein hormone. These have been obtained by X-ray analysis of insulin crystals.

In this discussion we will not attempt to give a full description of developments in experimental technique and mathematical analysis that made this advance possible. Instead we will attempt only to outline the problems that made the X-ray analysis of insulin less straightforward than that of many other proteins. This will allow greater discussion of the crystal structure itself. Also we will describe some of the features of the structure that appear relevant to an understanding of the chemistry and biology of insulin.

II. Preliminary Crystallographic Studies

Insulin was first crystallized in a rhombohedral form in 1926 by Abel. However, this could not be reliably repeated with purified insulin until D. A. Scott discovered that the presence of zinc ions was necessary for crystallization. He later experimented on the replacement of zinc by other metals and showed that insulin crystals could grow in the presence of iron, cobalt, nickel, and cadmium as well as zinc.

The first X-ray diffraction photographs of single rhombohedral crystals were taken by Crowfoot (1935) in Oxford. These showed that the crystals contained three equivalent units of weight about 12,000. When Sanger later determined the primary sequence of beef insulin (Ryle *et al.*, 1955), this was found to correspond with two insulin molecules. We now know that insulin crystallizes from aqueous solution in two rhombohedral forms which contain a minimum of two zinc ions and four zinc ions, respectively, per six insulin

¹ The Gregory Pincus Memorial Lecture.

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molecules; in the basic repeating unit, the rhombohedral cell, each form contains three equivalent insulin dimers related by a 3-fold axis. Figure 1 shows the rhombohedral 2 Zn insulin crystals (Schlichtkrull, 1956), and Fig. 2 an X-ray diffraction photograph. The 3-fold symmetry is apparent in both. Table I records the X-ray data on 2 Zn insulin crystals (Harding *et al.*, 1966).

The fact that insulin will also crystallize in other forms helped us to understand the symmetry of the packing of the insulin dimers and hexamers in the



FIG. 1. Rhombohedral 2 Zn insulin crystals.

rhombohedral crystals. Barbara Low and her colleagues have worked on zinc-free orthorhombic crystals obtained at low pH. They recognized that the asymmetric unit of these crystals contained two equivalent insulin monomers related by a 2-fold axis (Low and Einstein, 1960), and guessed that this might be so in the rhombohedral crystals as well. Later, using Rossman and Blow's functions, Dodson *et al.* (1966) were able to determine the position of this axis in the rhombohedral crystals. They also showed that a monoclinic form of zinc insulin, which crystallizes in the presence of phenol, contains six molecules in the asymmetric unit which has local 3-fold and 2-fold axes.

Thus, both the monoclinic and rhombohedral forms contain a hexamer of insulin, which has both 3-fold and 2-fold symmetry. In the 4 Zn rhombohedral crystals the 2-fold axes are in a plane perpendicular to the 3-fold axis but pass about 1 Å from this axis. In the monoclinic and 2 Zn rhombohedral forms, the

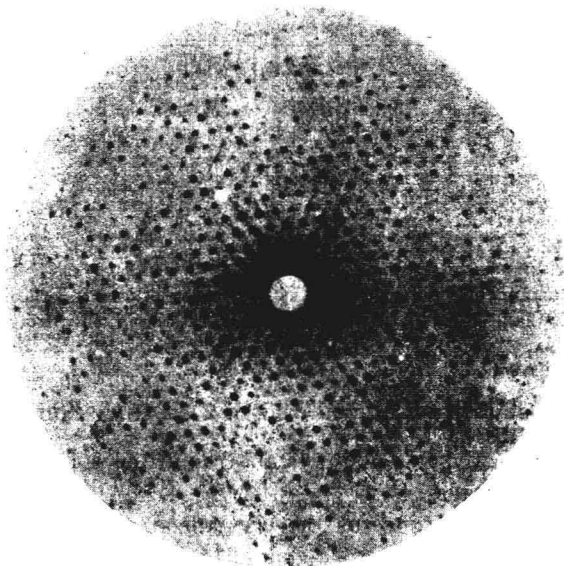


FIG. 2. X-Ray diffraction photograph (precession of $h k i 0$ zone) of rhombohedral lead insulin crystals.

TABLE I
*Unit Cell Dimensions and Solvent Contents of
2 Zn Insulin and 4 Zn Insulin*

Crystals	a_H (Å)	c_H (Å)	a_R (Å)	α_R	Solvent content (%)
2 Zn insulin	82.5	34.0	49.0	114.8°	30
4 Zn insulin	80.7	37.6	48.2	113.4°	34

2-fold axes not only are perpendicular to the 3-fold axis, but also intersect it. This arrangement has 32 symmetry and is shown in Fig. 3. The 2-fold axes are not crystallographic axes. They relate only those molecules within the hexamer, that is within the unit cell, and they are, therefore, local axes. It was an early and correct guess that the two zinc atoms lie on the 3-fold axis related by the 2-fold axes.

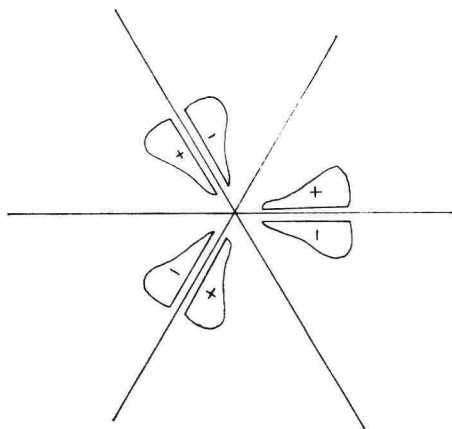


FIG. 3. The arrangement of insulin molecules in the hexamer. The 3-fold axis is perpendicular to the plane of the paper, and the 2-fold axes lie in this plane.

III. The Crystal Structure Analysis of 2 Zn Rhombohedral Insulin

The successful analysis of the crystal structure of a protein depends on three experimental stages.

1. The first of these concerns the crystallization of the protein. Large crystals, about 1 mm across, are required for an accurate analysis at high resolution. The very few accounts of hormone isolation and purification which describe good crystals indicate that this may be the main difficulty in the X-ray analysis of other protein hormones, but in the case of insulin, beautiful 2 Zn rhombohedral crystals of a suitable size were available. Most of our experiments have been carried out on pig insulin given to us by Dr. J. Schlichtkrull (Novo Terapeutisk Laboratorium, Copenhagen) and recrystallized according to methods described by him. We are very grateful to Dr. Schlichtkrull for the careful work that made the production of such crystals possible.

2. The second stage involves preparing crystals of the protein modified by the addition of atoms of high atomic weight. The conformation and packing of the protein molecules in the crystals of these heavy atom derivatives should be the same as those in the native crystals; in other words, they must be isomorphous. It was the demonstration of the use of the method of isomorphous replacement by Max Perutz nearly twenty years ago that ensured the success of protein X-ray analyses. With insulin, this procedure involved many difficulties.

Bernal realized that isomorphous replacement might be useful in the X-ray analysis of insulin in the very early days of protein crystallography, and he

suggested using Scott's cadmium crystals. However, the differences in the diffraction patterns of cadmium and zinc insulin crystals were small, and further work was not carried out at that time. More recently, Marjorie Harding and others made a careful study of the effect of heavy atom salts on insulin crystals and also attempted many cocrystallizations in the presence of these salts. The first breakthrough came in 1965 when it was discovered that the zinc ions could be removed from the rhombohedral crystals by using the chelating agent, EDTA. These zinc free crystals were stable enough to be removed for soaking in lead ions which became bound in a regular way. This derivative proved to be sufficiently isomorphous for a detailed analysis. However, the removal of zinc ions appeared to lead to systematic errors in the analysis, and in addition at least one other derivative was required for a complete determination by this technique.

The final success was the result of a further, very systematic study of the soaking of insulin crystals in heavy atom-containing solutions. Previously unsuccessful experiments were repeated with uranyl and uranyl-fluoride ions, and by careful control of temperature, concentration, and buffer, usable derivatives were prepared. It was also discovered that a different lead derivative could be produced by increasing the concentration of lead ions. A series of heavy atom-substituted aldehydes were also reacted with insulin in the hope that they would form Schiff's bases with the insulin α - and ϵ -amino groups. As a result of a great number of studies, it was found that mercurated metahydroxybenzaldehyde gave a reproducible, isomorphous derivative.

Thus, in 1969 we found ourselves with five isomorphous heavy atom derivatives. Each of them seemed usable, but we had serious reservations about all. Of the new derivatives, high lead concentrations damaged large crystals, the uranyl derivatives had disordered heavy atom positions, and the mercurated aldehyde preparations gave very low substitution. We can now see that these difficulties in forming good heavy atom derivatives mainly arose from the very close packing of the insulin molecules in the hexamer and of the hexamer in the rhombohedral lattice; the amount of solvent in the crystals of the rhombohedral form, 30–34% (Table I), is rather lower than for most protein crystals. Also the absence of reactive sulphydryl groups and the presence of an exposed disulfide bridge precluded the use of many of the reagents successful with other proteins.

3. The third stage involves the collection of X-ray data and the analysis of the X-ray intensities from the crystals of the native protein and heavy atom derivatives in order to derive the heavy atom positions and finally the electron density map of the protein.

The rhombohedral space group, R3, made this part of the analysis complicated. All crystals containing protein molecules formed from L-amino acids must be noncentrosymmetric. In fact, centrosymmetric crystals offer

great advantages in simplification of structure analysis, and it is fortunate that many protein crystal structures contain an evenfold axis of rotation which leads to an apparent centrosymmetric arrangement in projection. This is true for hemoglobin, lysozyme, ribonuclease, and other enzyme crystals so far studied, and it allowed straightforward exploratory studies when derivatives were being evaluated.

In the rhombohedral crystals of insulin, there is a 3-fold axis only; there is no evenfold crystallographic axis, and, therefore, there is no centrosymmetric projection. This meant that an alternative approach to analysis of the heavy atom derivatives was required. We achieved this by measuring an anomalous scattering effect which in noncentrosymmetric crystals results in small differences in the diffraction pattern. The combination of these small anomalous scattering differences with the larger isomorphous differences was the basis of our analysis. We acknowledge the work of Marjorie Harding and Margaret Adams, who were instrumental in originating and developing this approach. It was also an advantage to have an automated Hilger & Watts four-circle diffractometer which enabled us to measure the diffraction intensities both quickly and accurately. As we had to measure small differences accurately, we repeated our measurements several times and averaged the results. We collected data to a resolution of $2.8 \text{ \AA}$, and this involved recording about 60,000 intensities.

The interpretation of our X-ray data showed that the binding patterns in the lead and uranyl derivatives were very complicated. Nowhere did the ions occupy all the available equivalent positions in the crystal. The binding was characterized by a statistical distribution among five or more sites giving an average of about four metal ions per hexamer. The two lead derivatives had similar sites, but the increased concentration of lead ions in one led to an increased occupancy. The amino acid side-chains involved in the heavy atom binding sites can now be identified. In all cases, the heavy atoms are on the surface of the dimer; none penetrates the nonpolar core. Most of the metal ions are close to the carboxylate groups of one or more glutamate residues.

Despite the difficulties which were inherent in the individual heavy atom derivatives, the combination of the six isomorphous series gave a good electron density map at $2.8 \text{ \AA}$ resolution. A measure of the electron density is given by the average figure of merit, which was 0.8, and this led us to feel that the map was of good quality.

IV. The Electron Density Map

Parts of the electron density map are shown in Figs. 4 and 5. The electron density map was computed at intervals of about $1 \text{ \AA}$. Several sections perpendicular to the 3-fold axis are shown in a diagrammatic form in these figures.

The most obvious features in this 2.8 Å resolution electron density map are the two large spherical peaks on the 3-fold axis. These peaks represent the electron density of the zinc atoms; their positions are about 18 Å apart, and the two zinc ions per hexamer of insulin are in agreement with the finding of Schlichtkrull.

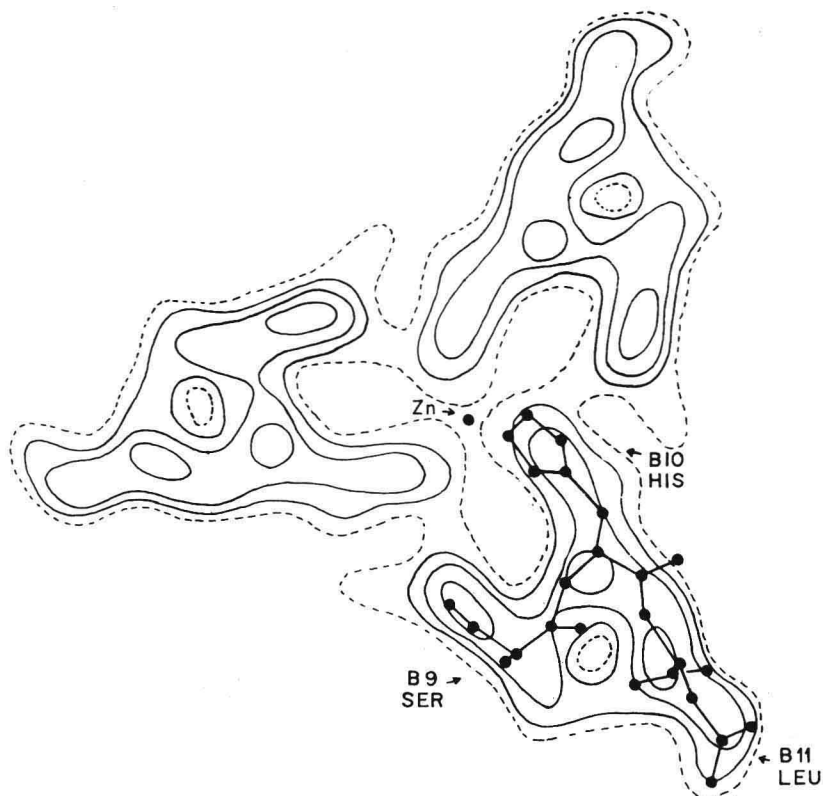


FIG. 4. The electron-density map at 2.8 Å resolution showing the appearances of residues B9, B10, and B11 of the insulin molecule. The section is taken perpendicular to the 3-fold axis. The atomic positions of one of the three equivalent groups are shown.

The electron density contains continuous chains, which are the polypeptide backbone of the insulin molecule. Most carbonyl oxygens are shown by small but well defined peaks of density in the chain, and the amino acid side chains also have density continuous with the backbone density. Figure 4 shows part of an α -helix. The side chains are those of B9 serine, B10 histidine, and B11 leucine, and the atomic positions are indicated for one of the equivalent positions. Figure 5 illustrates the appearance of other residues in the electron-

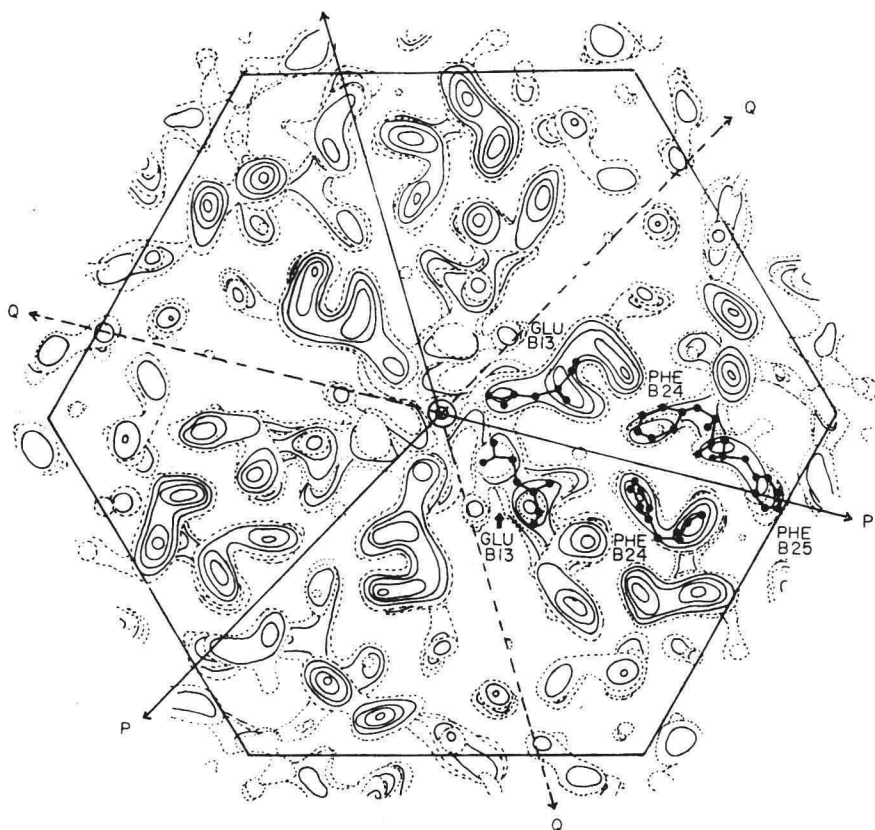


FIG. 5. The electron density map at 2.8 Å resolution showing the region of the 2-fold axes of symmetry (OP and OQ). The atomic positions of some selected residues are shown. The sections are computed perpendicular to the 3-fold axis.

density map. In particular, the rings of B24 phenylalanines are very well defined, as are the carboxylate ions of B13 glutamates.

Figure 5 also demonstrates another feature of the electron density map, and this is its symmetry. The 3-fold symmetry is evident. Also there are approximate 2-fold axes perpendicular to the 3-fold axis, marked OP and OQ. This arrangement of electron density agrees well with the predictions using the functions of Rossmann and Blow that have been discussed in Section II.

V. The Structure of the Insulin Monomer

The electron density map was interpreted initially from the hypothesis that the residue coordinated to the largest peak, the zinc atom, would be a