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*INSULIN—GROSS MOLECULAR STRUCTURE: TRIAL-AND-ERROR STUDIES USING TRANSFORM AND PATTERSON FUNCTION TECHNIQUES**

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X-ray crystal structure studies of the orthorhombic forms of bovine insulin have established: (1) the probability that there are rod-like regions of high electron density in these structures (these regions are not incompatible in kind with the assumption that they correspond to regions of α-helix configuration in the insulin molecule);[1-3] (2) that all the forms observed—wet, shrinkage stages, and air dried—are closely related, and that the molecular shifts between these crystal forms are principally simple translations along the three axes;[3-5] (3) that the structure of the insulin dimer is almost certainly essentially invariant in all the orthorhombic forms, and that the two molecules in the dimer are related by a twofold axis.[4, 5] More particularly, (*i*) in one shrinkage stage (type C), space group $I2_12_12_1$, the twofold axis is a crystallographic twofold axis;[4] (*ii*) in one of the two wet forms (type A), space group $P2_12_12_1$, the twofold axis (here noncrystallographic) is parallel or nearly parallel to a, and its approximate coordinates are: $y = 0.07$ and $z = 0.20$:[6] compare $y = 0$, $z = 0.25$ in $I2_12_12_1$. It follows from (2) and (3) that the marked pseudo-body-centered intensity distribution observed in the wet type B and the air-dried type P crystals arises from the condition that the dimer twofold axis in these structures is slightly displaced and/or tilted from its position in $I2_12_12_1$.

This study, an attempt to obtain information about the gross molecular structure of insulin by trial-and-error methods utilizing the Patterson function and Fourier-transform techniques, was undertaken as a possible complement to our work on the structure determination by the method of isomorphous replacement. The X-ray diffraction intensities of the four crystal forms A, B, C, and P show certain very striking features which are remarkably similar. Further, their Patterson functions suggested that these structures may be described, to a large extent, as made up of rod-like units oriented—at least in the B, C, and P forms—parallel or nearly parallel to a. The aim of this work was to define an arrangement of such units which would account for the main features of both Patterson functions and diffraction patterns.

Experimental.—The studies reported here were with insulin sulfate crystals. Most of the experimental data used were collected earlier.[1, 3, 4] The *hkk* reflections from type B crystals were recorded on film, and the intensities estimated visually; no absorption corrections were made. An optical diffractometer and a pantograph punch[7] were used in preparing the transforms.

Intensity Distributions and Patterson Functions.—The most striking feature of the weighted reciprocal lattices is a pattern of four high-intensity reflections, at spacings of about 10 Å, found in the 0*kl* planes (Fig. 1). This feature, which will be called the "cross pattern," is most marked in the C form. We have interpreted this pattern as the transform of an arrangement of subunits common (at least in projection) to all four forms. There is considerable evidence that the structure in the forms B, P, and C consists in large part of rod-like regions of electron density 10 Å apart: (1) The three-dimensional Patterson function for type P may be described as a close-packed array of rod-like regions of high vector density parallel to *a*, with a minimum spacing of about 10 Å in the direction of [012].[1] This array corresponds to the 10 Å "cross pattern." The *a*-plane Patterson projection for type C (Fig. 2) is very similar to that for type P.[1] (2) The *hkk* Patterson projection for type B (Fig. 3) shows lath-like regions of high vector density parallel to *a* with a separation of about 10 Å. (3) The *h*0*l*, *hk*0, and *hkk* Patterson projections for the type B crystals, considered together, show some evidence for a degree of cylindrical symmetry about the *a* axis, consistent with an array of approximately cylindrical rods oriented with their axes parallel or nearly parallel to *a*. Although there is no direct evidence for rod-like regions in the A form, the similarity of the 0*kl* projections of all four forms suggests common structural features.

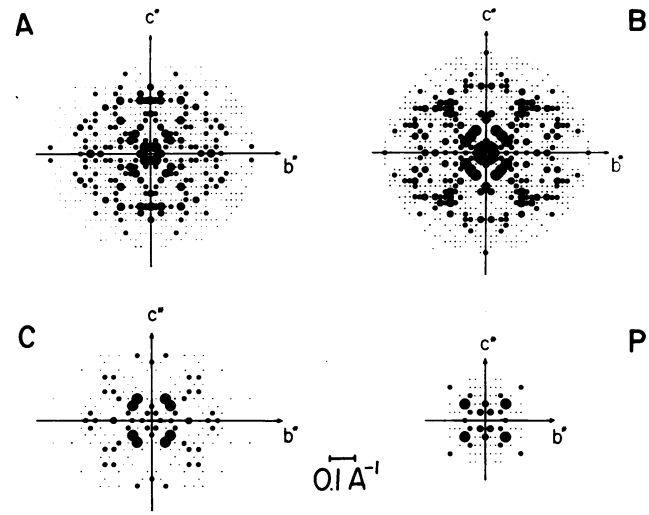

FIG. 1.—(0*Kl*) weighted-reciprocal-lattice sections for the A, B, C, and P crystal forms. F^2 values for the various crystal forms are not on the same scale (from J. R. Einstein and B. W. Low[4]).

A Model: Its Structural Implications.—In this work, we have made no assumptions concerning the conformations of those parts of the molecule in the rod-like regions of high electron density. None of the evidence is incompatible with the assumption that these may be regions of α-helix; indeed, the 10 Å separation would suggest this. Further, (1) all of the reciprocal-lattice sections containing the a* axis recorded for the B, P, and C forms show strong regions of intensity crossing this axis in the 5 Å region. This is consistent with the transform of an α-helix[8] tipped slightly with respect to the *a* axis. (2) In the *hkk* Patterson projection for the B form (Fig. 3), there are bands along the

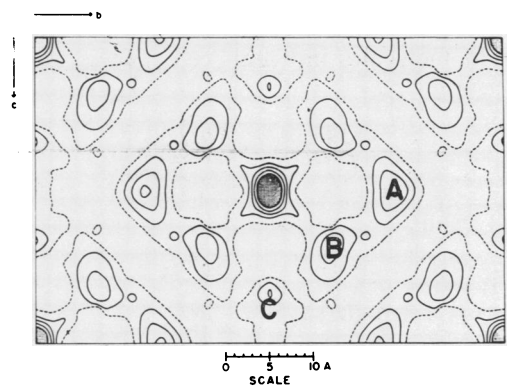

FIG. 2.—*a*-Plane Patterson projection for type C crystals. Contours are at equal arbitrary intervals. The dashed contour is the "zero" contour; "negative" contours are omitted.

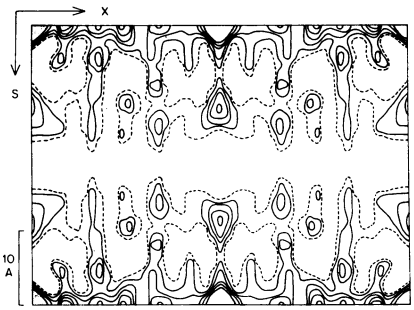

FIG. 3.—(*hkk*) Patterson projection for type B crystals. The letter *s* designates a vector lying in the *b*, *c* plane, and at an angle of approximately 41° to *c*. Contours are at equal arbitrary intervals. The dashed contour is the "zero" contour; "negative" contours are omitted.

laths of high vector density, which suggest a periodicity along *a* with a spacing of approximately 5 Å. If the *hkk*, *hk*0, and *h*0*l* Pattersons of the B form are placed origin to origin with *a* axes coinciding, then regions of high vector density overlap in such bands. (3) Studies of the optical rotation and rotatory dispersion of insulin solutions[9] and of deuterium exchange[10] have been interpreted as suggesting that a large part of the molecule may be in the α-helix configuration. The estimates, which range between 38 and 66%, correspond, for the optical rotatory dispersion studies, to excess right-handed helical content.

Consideration of the 0kl Weighted-Reciprocal-Lattice Sections: Use of Optical Transforms.—The early work was carried out before the finding of the C form. The "cross pattern" suggested a diamond array of units, each unit identified as the projection of a rod (Fig. 4*a*). Various arrangements of diamond arrays in the unit cells of types B and P crystals were tested by comparing their optical transforms with the 0*kl* weighted-reciprocal-lattice nets. In preparing the optical transforms, each unit of a diamond array was represented by a single circular hole in the diffraction mask. The centers of the diamonds were placed in positions suggested by the pseudo-symmetry, i.e., on or near the projections of one of the sets of special positions for space group $I2_12_12_1$, each of point group 2:

	$(0,0,0;\ 1/2,\ 1/2,\ 1/2)+$	Twofold Axis Parallel to
(1)	$x,\ 0,\ 1/4;\ \bar{x},\ 1/2,\ 1/4$	*a*
(2)	$1/4,\ y,\ 0;\ 1/4,\ \bar{y},\ 1/2$	*b*
(3)	$0,\ 1/4,\ z;\ 1/2,\ 1/4,\ \bar{z}$	*c*

The diamond was oriented with its diagonals parallel or nearly parallel to *b* and *c*. In each of these models there are four diamonds per unit cell, which represent the eight molecules in a unit cell. Thus, each molecule is represented (wholly or in

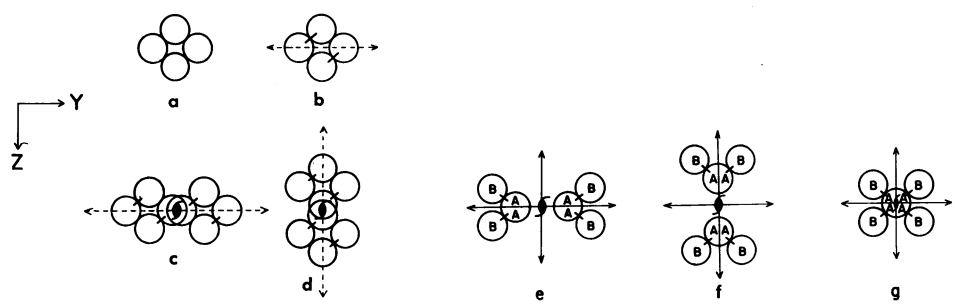

FIG. 4.—Models used in the optical transform studies of the 0*kl* data, (*a*) to (*d*) for the pseudo-body-centered crystal forms, (*e*) to (*g*) for the body-centered form C. The broken lines indicate pseudo-twofold axes. See text for discussion.

part) by two of the four units (rod-like regions) in a diamond array. The chemical bonding between the two units in a molecule is represented by a short line in Figure 4. A model, consisting of only two diamonds per unit cell, centered in projection at the positions $1/4$, 0, and $1/4$, $1/2$, is also consistent with the weighted reciprocal lattice and pseudo-symmetry. This model was initially considered unlikely; in projection, it corresponds to arrangement (1) discussed later.

The diamond position (1) always gave an unsatisfactory optical transform. In particular, it was never possible to bring the 011 reflection up to a reasonably high (relative) intensity. Optical transforms corresponding to positions (2) and (3) were both reasonably satisfactory when the diamonds overlapped as shown in Figure 4*c* and *d;* that of 4*c* gave the better agreement. The finding of the exactly body-centered C form invalidated these models: there can be only a pseudo-twofold axis in a diamond array placed in either position (2) or (3) (for example, dashed line in Fig. 4*b*).

All further work was carried out on the C form. This crystal form is clearly more favorable than the other forms for trial-and-error studies. A twofold rotation axis cannot intersect a polypeptide chain, nor the bounding surface of a model unit which represents a region of densely packed polypeptide chain. Thus, the 12 twofold rotation axes crisscrossing through the unit cell (space group $I2_12_12_1$) impose severe restrictions on the positions available for substantial lengths of rod-like structure parallel to *a* and of packing diameter about 10 Å.

Optical transform studies were made of two types of arrays: (*i*) pairs of units A + B overlapping to give a triangular array as in Figure 4*e* and *f*, B tentatively identified as a single rod and AA as two rods end-to-end in projection; (*ii*) pairs of units A + B in an overlapping arrangement as in Figure 4*g*, B tentatively identified as a single rod and AAAA as the overlapping projection of four units of unspecified structure. The unit A in Figure 4*g* cannot be identified as other than a rudimentary rod nearly parallel to *a*, since its length along *a* is at most a/4 or 13 Å. We therefore assumed no particular form for these units, but assumed merely that they are the parts of four molecules overlapping to give a high projected density at the 2_1 axis. The four units on the 2_1 axis form together, very roughly, a kind of interrupted rod-like region parallel to *a*. The common projection of these units was represented in the optical diffraction masks by a single circular hole. The arrangement which gave the optical transform in best agreement with the weighted reciprocal lattice was that of Figure 4*g;* this arrangement has much in common with those of Figure 4*c* and *d*, but the implied three-dimensional model is considerably different. The optical transform could be improved by representing B by an elongated hole, corresponding to a tilted rod (see Fig. 5*b*, in which a complete unit-cell projection for this arrangement is shown). The optical transform (Fig. 6*b*) then shows considerable agreement with the weighted-reciprocal-lattice net within the region $d \geq 8$ Å, with some agreement, perhaps fortuitous, at smaller spacings.

If the model is associated with the known primary structure of insulin, then the rod B probably represents the whole or a large part of the polypeptide chain B in the α-helix configuration. The presence of the intra-chain disulfide bond in the A chain prevents the whole chain from being coiled into one continuous α-helix. The unit A represents a small region of unspecified configuration in the A or B chain. A single molecule would then be represented by a rod B and one of a set of

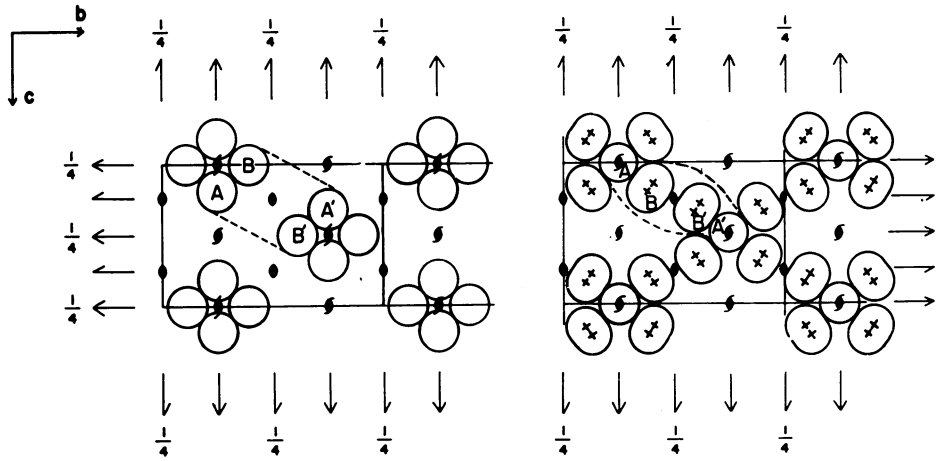

FIG. 5.—Unit-cell projections on the a-plane for two models considered. (a) Left: model corresponding to the point set of arrangement (1), Figure 7a. (b) Right: model corresponding to Figure 4g. In both (a) and (b), the dashed lines enclose a single insulin dimer ABB'A', as seen in projection.

four overlapping units A; illustrations of a three-dimensional arrangement of such a model are shown in Figure 8b and c.

Consideration of the Type C a-Plane Patterson Projection.—According to our hypothesis, the a-plane electron-density projection must be dominated by a few roughly circular regions, the rod-projections. The prominent peaks of the a-plane Patterson projection (marked A, B, C in Fig. 2) would then correspond primarily to inter-rod vectors. (The disparity in peak heights is minor, as $F^2(000)$ was omitted.) We have attempted to define all the possible models, the units of which are rod-projections and other limited dense regions, which would give reasonable agreement with the principal Patterson peak distribution. Two sets of positions have been defined; these are shown in Figure 7a and b, where each unit is represented, as an initial approximation, by a single point.

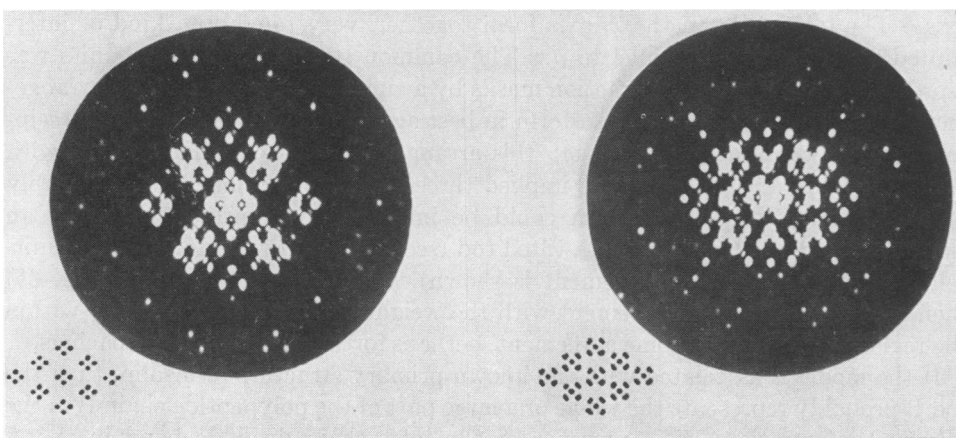

Fig. 6.—Optical transforms of the models of Figure 5. The masks from which the transforms were made appear at the lower left of each transform. (a) Left: transform corresponding to Figure 5a. (b) Right: transform corresponding to Figure 5b. These transforms are to be compared with the $0kl$ weighted-reciprocal-lattice section for the type C crystals (Fig. 1).

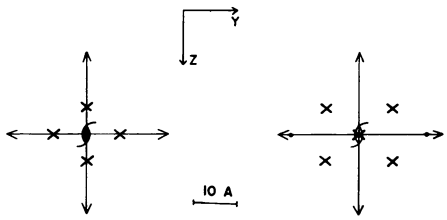

FIG. 7.—Two arrangements of points which give rise to vector sets corresponding to the a-plane Patterson projection for type C crystals. (a) Left: arrangement (1). (b) Right: arrangement (2). In arrangement (2), the crosses mark high-weight positions, and the dots, low-weight positions.

Arrangement (1) (Fig. 7a) is a point set which gives the precise vector set defined by the maxima of the Patterson projection. If each point is taken to represent the projection of a pair of rods, parallel and end-to-end, then the corresponding complete unit-cell projection is that shown in Figure 5a. This arrangement gives rise to an optical transform (Fig. 6a) which shows some agreement with the weighted reciprocal lattice.

If the model is associated with the known primary structure of insulin, then each rod would correspond to a part of one peptide chain. The limiting length for rods parallel to a is 26 Å (a = 52 Å). In terms of the α-helix configuration for the rods, this would correspond to a maximum of about 14 residues (21 Å) per rod, allowing for continuity with contiguous regions of peptide chain. A single molecule would then be represented by two α-helical segments (one per chain), parallel and adjacent to each other, but separated by a translation along their axial direction so that they lie side by side for a length of only five residues (see Fig. 8a). Although the rods may be tipped with respect to a and thus be longer, the equivalent substitution of pairs of elongated units for the circular ones of Figure 5a would not substantially improve the agreement with the Patterson projection.

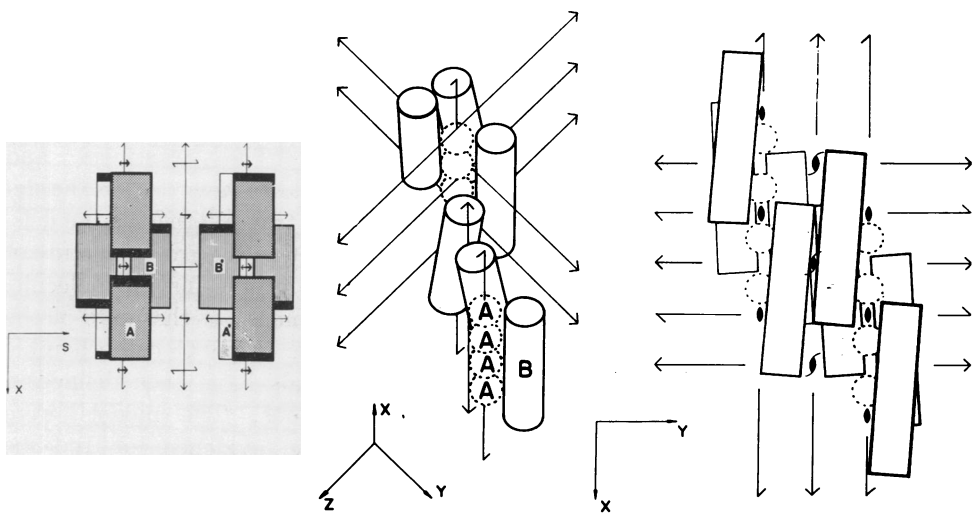

FIG. 8.—Three-dimensional illustrations of two models. (a) Left: model corresponding to arrangement (1), Figures 5a and 7a. The letter s designates the vector [011]. Both rod-like units A and B are arbitrarily assigned a zero tilt and a length of 21 Å. (b) Center and (c) right: two views of a model corresponding to Figures 4g, 5b, and 7b (low-weight positions omitted). The intersections of the units A, of unspecified gross structure, with the 2_1 axis are represented by dashed spheres, the centers of which are assigned a separation of exactly a/4 along the axis. Arbitrarily assumed parameters for the rod-like units B are: tilt, 6°; length, 45 Å; x-coordinates of rod centers, a/8 and symmetry-related positions.

Arrangement (2) (Fig. 7*b*) consists of high-weight positions (marked with crosses) and low-weight positions (marked with dots). The vector set of the cross positions contains one vector additional to those of the originally defined set. The terminus of this vector, however, lies within a positive region of the Patterson function, quite close to the broad peak A. There are also two new vectors, one of medium weight (cross to dot), which lies within a positive region, and one of low weight (dot to dot), which lies on the zero contour. This arrangement is essentially the one (Fig. 4*g*) found to be satisfactory in the optical-transform studies, with the addition of the low-weight units. The high-weight units will be designated A and B as in Figure 4*g*, and the low-weight units, C.

We have identified only unit B as a rod-projection: C has low weight (it could correspond to a short rod parallel to *a*); the restrictions on A have been discussed above. Assuming that units A and C are roughly circular in form, the agreement with the Patterson is improved if unit B represents a tilted rod, rather than one parallel to *a*. This may be simply shown by the calculation of a point vector set, using one point for a "circular" unit and two points for the projection of a tilted rod (see crosses in Fig. 5*b*). The new vector set is shown in Figure 9, superposed on the Patterson projection. The dashed lines indicate the expected ridges and plateaus which would be present in the vector structure of the model. The agreement with the Patterson shown here is better than any which could be obtained with arrangement (1). The final model of this arrangement, with the rods tipped, corresponds, if the low-weight units are ignored, to the model previously described (Figs. 5*b*, and 8*b* and *c*).

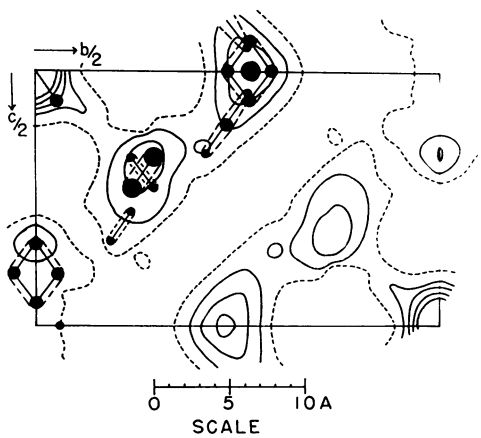

FIG. 9.—Vector set for the point set of arrangement (2), Figure 7*b*, where one cross position has been replaced by two as in Figure 5*b* (corresponding to a rod tilted with respect to *a*). The vector set is superimposed over the *a*-plane Patterson projection for the type C crystals. The points of arrangement (2) were arbitrarily weighted: cross position on 2_1 axis, 2; other cross positions, 1; dot positions, 1. The areas of the circles indicate the weights of the vectors.

Conclusion.—The transform and Patterson studies have led to the description of two alternative models, each representing the gross structure of a major part of the insulin molecule. The arrangements of the units of the models in the type C orthorhombic insulin crystals have been defined in two dimensions. The models were based on the evidence for rod-like regions of high electron density in the orthorhombic crystal forms of insulin. The vector structure study indicated that these models are the only two general models consistent with our basic hypothesis. This may be shown in another way. The positions in the *a*-plane projection of the rods would be indicated by the two very strong 10 Å reflections, 033 and 042, for which there are only two nonequivalent choices of signs, +,+ and −,−. These correspond, respectively, to arrangements (2) and (1).

Although the model corresponding to arrangement (2) showed a larger measure of

agreement with the observed data, both by optical transform studies and in the vector structure study, arrangement (1) was not clearly invalidated. Further studies involving refinements of the models have shown that if one of these two models is valid (that is, if the basic hypothesis is correct), then it must be the one corresponding to arrangement (2).[11]

* An earlier account of this work was presented at the Fifth International Congress of the International Union of Crystallography, Cambridge, England, in 1960; see published abstract (Low, B. W., A. S. McGavin, and J. R. Einstein, *Acta Cryst.*, **13**, 1056 (1960)).

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*CONTROL OF GLUCOSE OXIDATION IN ANTERIOR PITUITARY BY HORMONALLY SENSITIVE PYRIDINE NUCLEOTIDE TRANSHYDROGENASE**

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The hexose monophosphate pathway for the oxidation of glucose is present in anterior pituitary,[1] and, as will be pointed out later, it is highly probable that this pathway is responsible for a major portion of glucose metabolism by this tissue. The regulation of the hexose monophosphate pathway would therefore provide an important basis for the control of anterior pituitary activity. Barondes *et al.*[2] have recently reported that several compounds, including serotonin and epinephrine, stimulate the formation of $C^{14}O_2$ from glucose-1-C^{14} by slices of beef anterior