Insulin and Proinsulin Conformation in Solution

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SUMMARY

The physical chemistry of the insulin molecule in solution is reviewed. The nature of the self-association behavior is such that at physiological concentration levels insulin must exist as a monomer even in the presence of zinc. Analysis of the data on the interaction between zinc and insulin in solution leads to the conclusion that the zinc insulin complex formed has a conformation very similar to the x-ray structure of crystalline zinc insulin. Conformational studies on insulin and proinsulin support the following structural model—the insulin portion of the proinsulin molecule is in the same conformation as the free insulin molecule. Finally, studies indicate that the α-NH_2 group on the A_1 glycine of insulin is very important for biological activity. DIABETES *21* (Suppl. 2) :486-91, 1972.

The preceding papers in this symposium have dealt with the contributions that chemical approaches have made to our understanding of the molecular basis of action of the insulin molecule. This paper will attempt to describe the contributions that have arisen from conformational studies on the insulin and proinsulin molecules. The separation of chemical and physical approaches to structure-function relationships is, however, rather arbitrary. In practice, the two approaches are complementary and, in fact, must be used simultaneously. Otherwise, the assumption must be made that chemical modification of the insulin molecule involves no conformational change and that the changes in activity are a result only of the particular modification. Some groups in the molecule must function, however, to establish the three-dimensional structure that is needed for full biological activity. Thus, modification of these non-"active-site" groups could cause conformational changes and consequently activity changes.

In the last thirty to forty years, the physical chemist has studied few proteins as extensively as he has insulin. This has resulted in the generation of a large amount of physical data on this molecule. This review, therefore, will concentrate mainly on the data that give insight into the problems of the state of insulin at physiological concentration levels, the nature of the interaction of zinc with insulin in solution and, finally, the conformational relationship between insulin and proinsulin.

The conclusions reached on the conformation of insulin in solution are in good agreement with the structural information available from the x-ray crystallography studies on zinc-insulin crystals.

State of insulin at physiological concentration levels

Both before and since Sanger's elucidation of the amino acid sequence of insulin,[1] a variety of physical technics have been used to determine the molecular weight of insulin in solution. All of these studies have shown that insulin self-associates quite strongly. In general, the aggregation behavior is a function of temperature, pH and ionic strength as well as protein concentration. The early studies of Steiner[2] and more recently those of Jeffrey and Coates[3] established that insulin solutions are a heterogeneous mixture of even aggregate states, e.g. dimers, tetramers, hexamers, etc., all in equilibrium with insulin monomer. The equilibrium constants for the equilibria between these different association states were determined, but unfortunately these studies were done in acid solutions (pH 2). At neutral pH, the association behavior becomes much stronger; molecular weights as much as twelve times the monomer molecular weight have been measured (see figure 1). With recent developments in ultracentrifugation technics, however, more precise measurements can be made at low insulin concentrations. Studies in our laboratory[4] and by Goldman[5] have shown the presence in solution of appreciable amounts of monomer at total insulin concentrations below 100 μg./ml. at neutral pH. Calculations using the equilibrium constants (see table 1) determined in these studies show that at physiological concentration levels ($\backsim$1 ng./ml.) insulin will be in essentially

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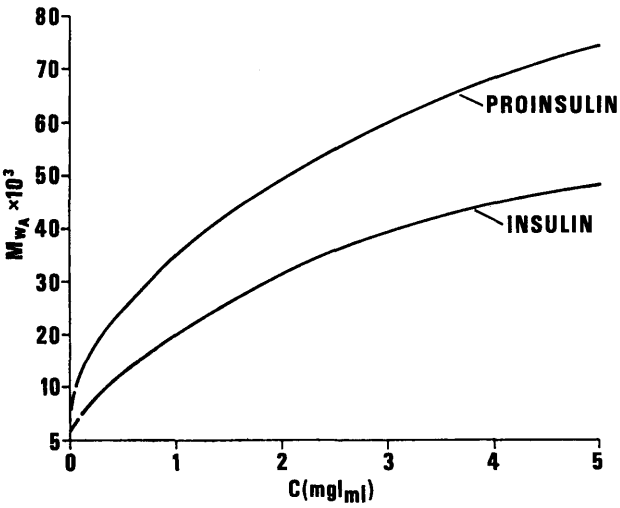

FIG. 1. Observed molecular weight as a function of protein concentration for insulin and proinsulin at neutral pH.

a monomer form only. The studies of Cuatrecasas[6] on the activity of insulin covalently linked to Sepharose also apparently support the conclusion that the monomer is the active form of the insulin molecule.

Another aspect of the self-association behavior is the determination of the residues in the molecule that are involved in the association reactions. The most substantial evidence has been accumulated on the dimer formation step. Using ultraviolet difference spectroscopy, Rupley et al.[7] demonstrated that phenylalanine and tyrosine residues undergo changes in their environment when

TABLE 1
Insulin self-association constants

pH	K dimer (M^{-1})	Reference
2	8×10^3	3
7	2×10^5	4
8	2×10^5	5

the dimer is formed (see figure 2). Goldman[5] and Ettinger and Timasheff[8] have shown a concentration-dependent circular dichroic (CD) effect in insulin solutions which is also due to a tyrosine residue becoming more rigidly oriented. This effect also correlates with insulin dimer formation. Finally, Goldman,[5] Carpenter,[9] and Menendez and Herskovits[10] have shown that desoctapeptide insulin does not exhibit the association behavior of the intact insulin molecule. Thus, the Phe-Phe-Tyr (B_{24} to B_{26}) residues are most likely involved in the hydrophobic interaction that leads to insulin dimers. This interaction between monomer units is also quite apparent in the x-ray structure of insulin. In connection with the desoctapeptide insulin, we would note that although this derivative has very little biological activity, circular dichroic studies[9,10] indicate the molecule has undergone a large conformational change. Therefore, it is not possible to conclude that any of the residues in the B_{23} to B_{30} region of the molecule are part of the active site of the insulin molecule.

Very little can be said concerning what regions of the insulin molecule are the interaction sites for the higher aggregation states. Examination of the x-ray crystal structure enables some speculation on the nature of the higher aggregation sites, but to this author's knowledge no studies have been carried out that have been specifically designed to gain information on these sites.

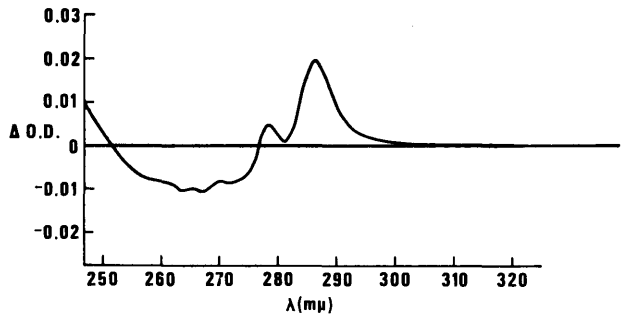

FIG. 2. Ultraviolet difference spectrum observed when zinc is added to insulin solution. Reference cell contains insulin at the same concentration as the sample cell. Sample cell contains 1 gm.-atom zinc per 2 moles of insulin.

Interaction with zinc

The characteristics of the interaction of zinc with insulin were examined early by Cunningham et al.[11] and Fredericq,[12] and more recently by Goldman[5] and by Coombs et al.[13] These studies have shown two types of zinc binding sites in the insulin molecule. The first of these is a strong binding site ($K \backsim 10^5$ to 10^6) while the second is a group of weak binding sites ($K \backsim 10^3$). Calculations made using these binding constants show that at physiological concentrations the zinc insulin complex is completely dissociated into monomers. Experimental demonstration of the dissociation of this complex has been made.[4,5] Tanford and Epstein[14] concluded from comparative titration studies on zinc-free and zinc insulin that the strong zinc binding site involved either the B_5 or B_{10} histidine residue. Weil et al.[15] have provided support for this conclusion through studies on the photooxidation of the insulin molecule. The x-ray structure shows the zinc is complexed with the B_{10} histidine. Goldman has shown that the amount of zinc bound by insulin requires the formation of

hexamers and is a function of pH. At acid pH, insulin does not bind zinc. Over a pH range of about 5 to 7, only the strong binding occurs. Above pH 7, the weaker sites start to bind zinc. The general pH characteristics of this binding indicate that the weaker binding sites probably include the α-amino groups in the molecule.

The zinc-insulin complex formed in solution consists of six (6) insulin molecules with a minimum of two (2) zinc ions. This complex corresponds quite well to the primary unit in the zinc-insulin crystals used for the x-ray analysis. A number of changes in physical parameters can be observed when zinc is added to insulin solutions. Some of the most prominent are a marked increase in the negative circular dichroic band at 275 mμ., generation of an ultraviolet difference spectrum similar to that seen for insulin dimer formation, and a marked change in the titration behavior of the tyrosine residues in the protein. The CD change observed upon addition of zinc certainly must indicate the strong orientation of one tyrosine residue per insulin molecule in the hexamer complex (see figure 3). The orientation of this

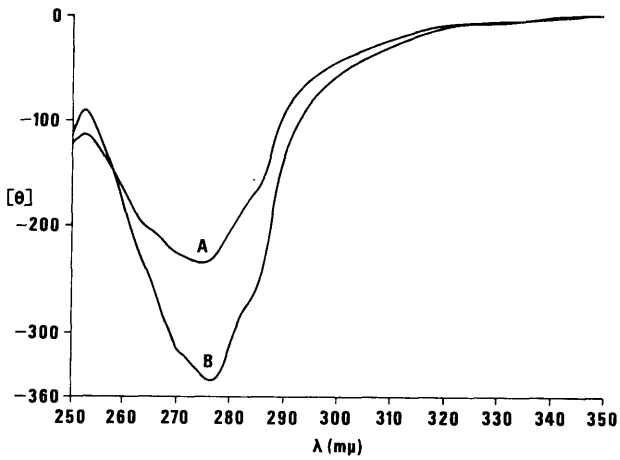

FIG. 3. Near ultraviolet CD spectrum of insulin without zinc (A) and with zinc (B). Insulin concentration of 1 mg./ml. at pH 7.4. Zinc concentration in solution for lower curve of 1 gm.-atom zinc per 2 moles of insulin.

residue apparently does not occur in the zinc-free insulin aggregates. Since the B_{26} tyrosine residue is already oriented in the insulin dimer, one of the other three tyrosines must be the source of this effect. Chemical modification studies[16,17] suggest that the B_{16} tyrosine is buried or in an inaccessible region of the molecule, and that tyrosine A_{19} is free in both zinc-free and zinc insulin. The same studies also suggest that A_{14} tyrosine is involved in an interaction in zinc insulin and not in zinc-free insulin. Thus, the observed zinc CD effect is probably an indication of orientation of the A_{14} tyrosine when the complex is formed. Again the x-ray crystal structure provides good confirmation of this conclusion.

A number of other studies have been done in order to establish the state of the tyrosine residues in insulin. Menendez and Herskovits,[10] using ultraviolet solvent perturbation spectroscopy, have suggested that zinc and zinc-free insulin have one free, one partly buried and two completely buried tyrosine residues. Tyrosine titration studies also have been done to establish the state of these residues. The interpretation of the results of these studies unfortunately is confusing because a number of factors which affect the titration behavior have been overlooked. As a part of our conformational studies on proinsulin, we have reexamined the titration behavior of the tyrosine residues in insulin. Beyond the normal effect of ionic strength which one would expect to be present, protein concentration, cleavage of disulfide bonds, and the presence of zinc markedly affect the titration behavior. Titrations performed in low ionic strength solvents (μ <0.1) result in a depression of the ionization due to charge effects (see figure 4). Unfortunately, these are the conditions of the original classical studies of Crammer and Neuberger.[18] If the titration is done at ionic strengths high enough to remove the charge effects, there is a slow time-dependent change in the UV difference spectrum at pH's greater than 11.5 (see figure 5) which is due to the cleavage of disulfide bonds and subsequent chemical reactions.[19-21] These effects are found either in the presence or absence of zinc at all insulin concentrations. At higher insulin concentrations (10 mg./ml.), the ionization of the tyrosines is somewhat depressed (see figure 6). This is a consequence of

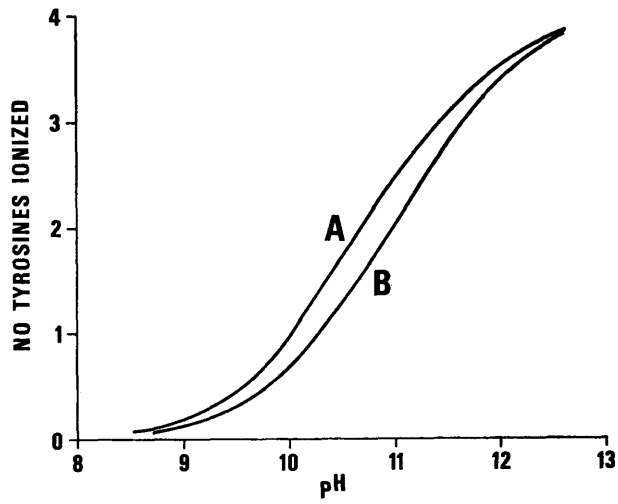

FIG. 4. Effect of ionic strength on the tyrosine titration curve of insulin. Upper curve solution contains 0.1M KCl. Lower curve solution contains 0.01M KCl.

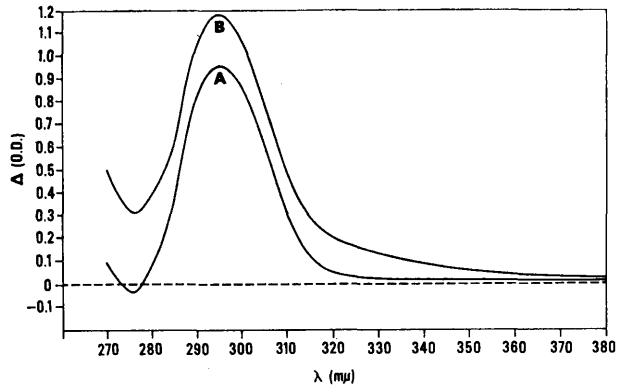

FIG. 5. Insulin ultraviolet difference spectrum. Reference cell contains insulin at neutral pH. Sample cell contains insulin at pH 11.5. Curve A was recorded three minutes after adjusting pH to 11.5. Curve B was recorded two hours after curve A.

the fact that even at pH 10 insulin still self-associates in these concentrated solutions. An analogous effect has been observed in glucagon solutions.[22]

The effect of zinc on the titration of the tyrosine residues is even more striking. A marked initial depression of the tyrosine ionization occurs in insulin solutions containing one gm.-atom zinc per 2 moles of insulin, e.g. at pH 11, in zinc-free insulin 2.5 tyrosines ionize, while in zinc insulin only 1.5 tyrosines ionize *initially* (see figure 7). However, an additional tyrosine rapidly ionizes so that after a short time period the same number of tyrosines have ionized in both the zinc-free and zinc insulin molecules. This phenomenon probably occurs because there are tyrosine residues buried in the zinc complex which become exposed as the complex dissociates and the individual insulin molecules unfold. This problem is currently under investigation in our laboratory.

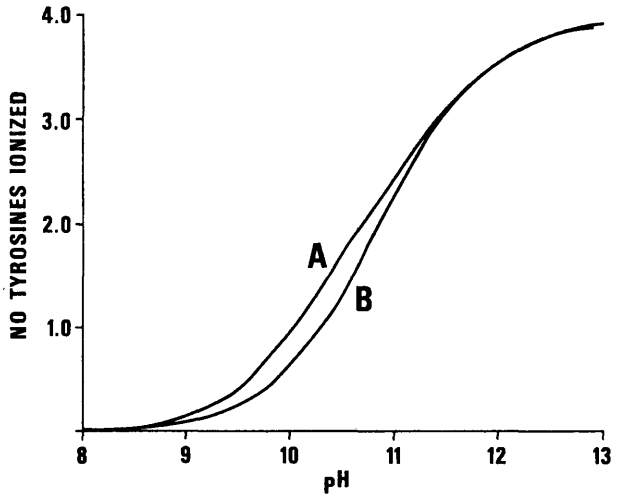

FIG. 6. Effect of insulin concentration on the tyrosine titration curve. Curve A is for 1 mg./ml. insulin. Curve B is for 10 mg./ml. insulin.

Conformational relationship of insulin and proinsulin

Attempts to determine the peptide backbone conformation of the insulin molecule have centered mainly about studies using optical rotatory dispersion (ORD) and circular dichroism (CD). A number of calculations of the per cent α-helix in the insulin molecule have been made based on analyzing the low ultraviolet ORD and CD spectra.[23,24] Most of these calculations predict about 30-40 per cent α-helix in the protein. Though this value is in general agreement with the amount of helix found in the x-ray structure, the agreement is most likely fortuitous since such calculations involve a number of very questionable assumptions. For example, one assumption is that α-helix and random coil are the only types of structure present in the molecule. Moreover, none of these calculations takes into account the aggregation behavior of the insulin molecule.

Our laboratory has been attempting to determine the conformation of proinsulin through examining its physical properties[25,26] The similarity in the physical properties of proinsulin and insulin is very striking. Proinsulin, like insulin, self-associates strongly over a wide range in pH. The types of aggregates as well as the self-association constants are essentially identical for both proteins at acid and neutral pH. Proinsulin exhibits the same kind of concentration dependency of its near ultraviolet and circular dichroic spectra as was described earlier for

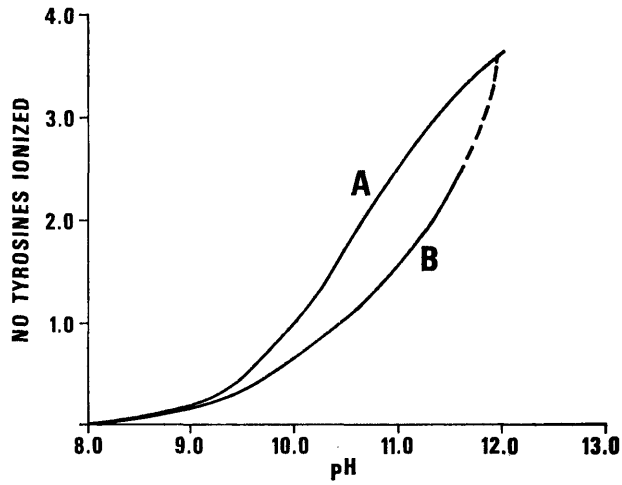

FIG. 7. Effect of zinc on the tyrosine titration curve. Curve A is insulin solution with no zinc. Curve B is insulin solution with 1 gm.-atom zinc per 2 moles of insulin. The $\triangle O.D._{295}$ shown in curve B was determined fifteen seconds after addition of NaOH to bring pH to the indicated value.

insulin. The titration behavior of the tyrosine residues in proinsulin is the same as that discussed before for the insulin tyrosine residues. Proinsulin interacts with zinc to form a complex which consists of 2 gm.-atoms of zinc and 6 proinsulin molecules (molecular weight of the complex is 55,000). Moreover, the strong zinc-binding constant for proinsulin is essentially the same as that found for insulin.[13] Proinsulins from different species (pork and beef) also exhibit the same physical properties.

On the basis of these data, we have previously proposed a model for the conformation of the proinsulin molecule. In this model we suggested that the insulin portion of the proinsulin molecule existed in essentially the same conformation as the free insulin molecule. Some additional experiments have been performed which strongly support this model of the proinsulin molecule. First, the CD spectrum of an equimolar mixture of insulin and the 31-residue connecting-peptide fragment from pork proinsulin was determined and was (within experimental error) equal to the CD spectrum of the intact proinsulin molecule (see figure 8). The second experiment was to monitor the tryptic transformation of proinsulin to desalanylinsulin by CD spectroscopy and acrylamide gel electrophoresis. No change from the initial CD spectrum of the proinsulin solution was found even when more than 90 per cent of the proinsulin had been converted to desalanylinsulin. We should also note that we have not found evidence for binding of the connecting peptide to insulin molecule in these studies.

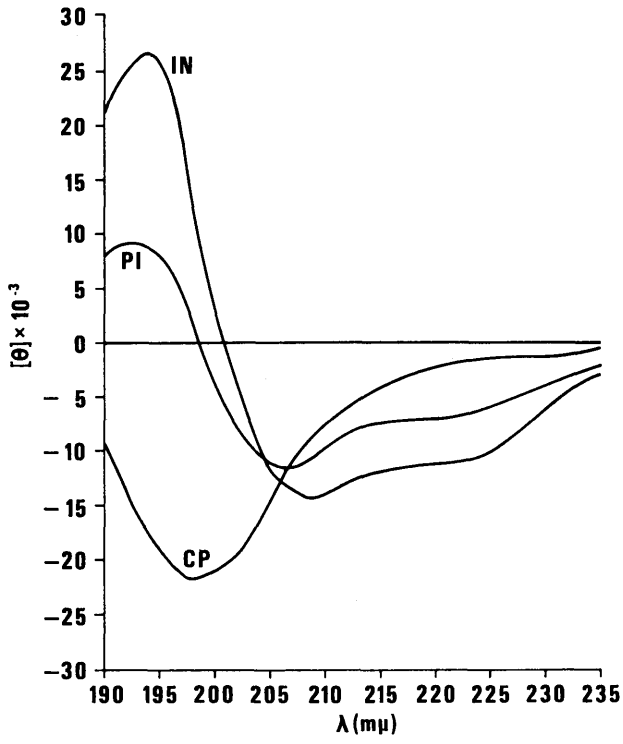

FIG. 8. Circular dichroic spectra of insulin (IN), proinsulin (PI), and 31-residue connecting peptide (CP) at neutral pH.

TABLE 2
Biological activity of proinsulin and proinsulin intermediates[27] (mouse convulsion assay)

Compound	Per cent activity (units)
Proinsulin	17
54-55 Split proinsulin	20
Desnonapeptide proinsulin	62
B_{31-32} diarginyl insulin	61
Insulin	100

We have also examined the CD spectra of some of the proinsulin "intermediates" discussed earlier by Dr. Chance—B_{54-55} split proinsulin, desnonapeptide proinsulin and B_{31-32} diarginylinsulin. The CD spectra of the 54-55 split proinsulin and the desnonapeptide proinsulin both are such that we have concluded that the insulin moiety is in essentially the same conformation in these "intermediates" as in the free insulin molecule. The biological activities of proinsulin and the 54-55 split proinsulin are both lower than the activity of desnonapeptide proinsulin (see table 2). Therefore, we conclude that the α-NH_2 group of the A_1 glycine is an important part of the "active site" of the insulin molecule. The insulin moiety relationship does not appear to be valid in the case of the diarginylinsulin intermediate. There is a larger decrease in the 195 mμ. CD band than can be accounted for just by the addition of the two amino acid residues ($[\theta]_{195}$ decreases from 27,000 to 18,000). Thus, a conformational change in this molecule may account for its diminished biological activity.

CONCLUSIONS

A good deal of information on the conformation of insulin has been derived from the physical studies discussed. Most importantly, this information (especially that for the zinc insulin complex in solution) appears to be in excellent agreement with the structure of insulin as derived from the x-ray crystallography data.

The physical data discussed in this review leave little doubt that at physiological concentration levels, either in the presence or absence of zinc, insulin exists as a monomer (molecular weight 5,800).

The conformational relationship between insulin and proinsulin is that the insulin moiety of proinsulin exists in the same conformation as the free insulin molecule. This relationship in combination with biological activity data indicates a role for the α-NH_2 group of the A_1 glycine residue in the activity of the insulin molecule.

Thus these physical studies have provided an independent route to the same conclusion as was reached by the semisynthetic chemical approach discussed in this symposium by Dr. Zahn.

We would like to speculate on some different aspects of structure-function relationships in the insulin and proinsulin molecules. These may be pertinent not only to structure-biological activity relationships but to the other stages of the in vivo existence of these protein molecules. We have previously suggested[13,25] that the interaction of proinsulin with zinc to form a specific complex may have a functional role in controlling the transformation process and therefore will not discuss this further here.

Let us now consider the point that insulin exists most likely in a monomer form in serum. The question then arises, why should this protein exhibit such strong and specific self-association behavior in addition to its interaction with zinc? We suggest that the functional role of the self-association behavior is to insure the efficient storage of this protein. If this is the case, then there would be a need during evolution to conserve certain amino acid residues in order to have the proper interactions in the insulin aggregates. Thus a number of residues, such as the B_{24} to B_{26} sequence which is involved in dimer formation, may have been conserved for the storage function and not necessarily for activity purposes.

The physical studies on insulin and proinsulin have yielded some insight into the relationship of the structure of insulin to its biological activity. We further suggest that our knowledge of the physical properties of insulin and proinsulin is equally applicable to understanding other biological phenomena such as the biosynthesis, transformation and storage of insulin.

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