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Biological Activity of Insulin-Sepharose?

In a series of independent studies pertaining to the mechanism of action of insulin (*1*) and to altered sensitivity to insulin in both diabetes (*2*) and obesity (*3*), Cuatrecasas and co-workers reported the common premise in these studies to be that insulin elicits its biological actions by interacting with specific receptor sites located on the cell membrane. The key reference cited in each report (*1–3*) to justify this basic premise is a study by Cuatrecasas published in 1969 (*4*). In that study (*4*), evidence was presented which purportedly demonstrated retention of the hormonal activities of insulin covalently coupled to insoluble beaded agarose (Sepharose) polymers. On the basis of the relatively large size of the insulin-Sepharose bead as compared to the target intact fat cell, he concluded that the insulin molecule could not enter the intact cell and that therefore all of the metabolic effects of the hormone resulted exclusively from specific interactions with the cell membrane (*4*). The acceptance of this evidence as experimental confirmation of the unitary view of insulin action, in addition to the recent proliferation of studies on cell membrane insulin-binding sites attest to the importance that has been attached to these conclusions (*5*). However, calculations performed on the data taken from the original Cuatrecasas report (*4*), and developed in this comment, indicate a discrepancy that seriously questions the validity of the insulin-Sepharose results. Recent studies on insulin-Sepharose (*6, 7*) previously have cast some doubt on the interpretations of such experiments.

By calculating the amount of insulin coupled per bead of Sepharose, and comparing this with the amounts of immobilized insulin that were reported to be bioassayed (*4*), it appears that the total amounts recorded as assayed were, in many cases, less than the amounts of insulin coupled per bead. It therefore becomes questionable as to how this was operationally possible. As stated in table 1 of reference (*4*), the insulin-Phe-Sepharose and insulin-Lys-Sepharose (*8*) used in the study contained 320 and 360 μg, respectively, of crystalline zinc insulin coupled per milliliter of packed Sepharose. The lowest concentration reported for any other preparation was 171 μg/ml. We have been able to confirm that these concentrations of insulin bound to Sepharose indeed do result from the described conditions.

As was stated in that report (*4*), the Sepharose beads used ranged in particle size from 60 to 300 μm. This coincides with the range for Sepharose 2B in the swollen state (*9, 10*). Therefore, calculations of the maximum, minimum, and average number of beads theoretically capable of occupying a cubic centimeter can be made. We calculate (*11*) the maximum number that could theoretically be squashed (forced) into this space to be about 10^7 beads, based upon a spherical volume of $(4/3)\pi r^3$ per bead and a minimum diameter of 30 μm per bead. The latter value represents half of the minimum size for any of the available Sepharose preparations, according to determinations of the size distributions by the Pharmacia Company (*10*). The minimum number of beads per cubic centimeter would be about 4×10^4, assuming a maximum average size of 300 μm per bead and natural (unforced) sedimentation. On the basis of a "harmonic average" size of 163 μm per bead (*10*), there would be less than 10^6 beads per cubic centimeter of packed Sepharose. According to experimental evidence documented by the Pharmacia Company (*10*), sample batches of settled Sepharose 2B contain about 3.24×10^5 particles per milliliter. Neither we (*12*), nor the Pharmacia Company (*10*), could find any significant effects of cyanogen bromide treatment or insulin coupling on the particle size range of the Sepharose beads.

Therefore, on the basis of an activity of 25 international units per milligram of native crystalline insulin (used to convert micrograms to microunits), there would be from 0.85 to 200 microunits of insulin bound per bead of insulin-Sepharose. These values are derived from an average of 340 μg of insulin reported by Cuatrecasas to be coupled per milliliter of Sepharose for the insulin-Phe-Sepharose and insulin-Lys-Sepharose preparations. Thus, when the most reasonable number (about 5×10^5) of beads is assumed to be present per milliliter of Sepharose (which is derived from the harmonic average of the particle size distribution), there must be more than 10 microunits of insulin bound per bead.

According to Cuatrecasas (*4*), the insulin-Phe-Sepharose and insulin-Lys-Sepharose preparations had potencies, on intact fat cells, virtually equivalent to that of free native insulin on the basis of conversion of [^{14}C]glucose to $^{14}CO_2$, fatty acids, and glyceride glycerol and of suppression of hormone stimulated lipolysis. Yet, in all of these bioassays, in vitro, the total amounts of insulin-Sepharose reported to be present in the final incubation media [tables 4 and 5 and figure 2 in reference (*4*)] ranged from 2 to 2000 microunits. Most of the assays were conducted on insulin-Sepharose reported in total amounts ranging from 2 to 60 microunits per final incubation volume. Thus, in order to provide these insulin-Sepharose concentrations, only a few beads, and in many cases only a fraction of a single bead, would have had to have been present in the incubation vessels. Assuming the extremely unlikely circumstances that there was only the calculated minimum amount (0.85 microunits) of insulin associated with an average bead, there still would have had to have been no more than a relatively small number of beads present. Since it is likely that these adipocyte assays utilize about 10^4 to 10^5 cells per incubation volume cell size [50 to 100 μm (*4*)], the significance of any metabolic effects of such small numbers of beads (in most cases less than ten) would be subject to serious question. In an attempt to repeat the experiments of Cuatrecasas, one is faced with the

dilemma of explaining how it would be operationally possible to dilute a suspension of insulin-Sepharose equivalent to about 17 microunits of insulin immobilized per bead (calculated from 340 μg of insulin coupled per milliliter) to a range of from 2 to 10 microunits of insulin-Sepharose per final volume.

Possibly some fragmentation of the beads, or elution (solubilization) of free insulin from the beads (*7*), could have occurred. However, in order to explain the nearly identical specific activities reported for the free, compared to immobilized, insulin, such fragmentation or elution would have had to have been virtually total. That complete fragmentation of Sepharose could have occurred under the described conditions (*4*) has been considered extremely unlikely (*10*). In any case, such extensive fragmentation would only enhance the likelihood of phagocytosis or solubilization of insulin and would be inconsistent with the conclusion (*4*) that insulin-Sepharose, by virtue of its large size, could not enter the cell. It also may be considered possible, but again extremely unlikely (*6*), that every molecule of insulin immobilized per bead is biologically more active than, and as accessible to the cells as, free insulin. However, such a consideration would further contradict the results inasmuch as it would require the assay of less rather than more beads.

It must be pointed out, however, that these comments are in no way meant to imply that insulin cannot be successfully coupled covalently to Sepharose with retention of hormonal activity. Nor are these conclusions meant to conflict with the unitary view that the metabolic effects produced by insulin result from the propagation of effects initiated exclusively from specific interaction of insulin with the cell membrane. However, it is intended to point out that the utilization of immobilized insulin by the procedures previously described (*4*) apparently has not yet provided the demonstration necessary to confirm these hypotheses. Accordingly, these calculations also demonstrate a previously unrecognized problem that now must be considered in the bioassay of significant amounts of any biologically active ligand coupled to insoluble particles.

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Katzen and Vlahakes' calculations are erroneous for the simple reason that they assumed that the insulin-agarose derivatives used for the biological assays were the identical ones which were used to characterize the chemical linkage form of the derivatives (*1*). The chemical studies (enzymic digestions, sulfitolysis, amino acid analyses, and so forth) obviously require much higher concentrations of insulin than do the biological studies. The three different types of derivatives used in the metabolic studies were prepared under the *same conditions* (*p*H, amount of CNBr, acetylinsulin) as those used for the chemical studies, but the amounts of insulin added and coupled were very much smaller since it obviously would otherwise not be possible experimentally to test very low concentrations of insulin-agarose on cells. By the same procedures numerous derivatives have been prepared (for these and other studies) which contain from 20 ng to 1 μg of insulin per milliliter of agarose.

Table 1. Effect of insulin coupled to poly-L-lysine-poly-L-alanine agarose on glucose oxidation by isolated fat cells. In experiments A through E, fat cells were incubated (*1*) at 37°C for 60 minutes with [^{14}C]glucose (0.4 m*M*, 9 μc per micromole) and native insulin or insulin bound to the branched copolymer, poly-L-lysine-poly-L-alanine, attached to agarose. Identical samples incubated (37°C, 60 minutes) in the absence of [^{14}C]glucose were centrifuged and the cell- and agarose-free medium from these incubations was used with fresh cells and [^{14}C]glucose to detect free insulin (experiments G to J). Abbreviations: Amt., amount; Conv., conversion of [^{14}C]glucose to $^{14}CO_2$.

Experiment	Incubation		Conv. (count/min)
	Addition	Amt. (μunit/ml)	
A	None		8,300
B	Insulin	5	19,400
C	Insulin	200	64,800
D	Insulin-agarose	100	52,200
E	Insulin-agarose, trypsin-treated	400	10,400
G	None		7,100
H	Medium of B above		15,300
I	Medium of C above		59,800
J	Medium of D above		8,400

Katzen and Vlahakes are also inaccurate in stating that the insulin-agarose studies have been interpreted as "experimental confirmation of the unitary view of insulin action." The studies were simply interpreted as indicating "that interaction of insulin with cell surface structures is sufficient to initiate a variety of metabolic alterations" (*1*). The studies or the interpretations do not exclude the possible existence of multiple and heterogeneous receptors in the cell membrane, or even the existence of additional, special intracellular receptors.

Others have also utilized insulin-agarose derivatives to elicit various kinds of metabolic effects in a variety of cells (*2*). Of particular interest are the studies of Oku and Topper showing that immature mammary gland cells, which are insensitive to native insulin, respond nicely to insulin-agarose. Furthermore, since the latter effect is blocked by native insulin, it is not possible that the insulin effects result from leakage of free insulin into the medium. Equally important are the recent reports demonstrating that large polymers of dextran containing covalently linked insulin are biologically active (*3*); such derivatives in fact appear to be more potent than native insulin. There are now many reasons for believing that the mechanisms by which insoluble insulin polymers activate insulin responses may differ from those of native insulin (*2*, *4*). To further understand the mechanism of insulin action it may be more fruitful to explore experimentally some of these exciting possibilities than to dwell on peripheral issues.

In questioning the hypothesis that insulin interacts with specific membrane-localized receptors Katzen and Vlahakes inexplicably ignore a large body of inseparable experimental data which has emerged during the past 2 years from a number of different laboratories. Highly specific binding structures (receptors) have been identified

and studied in intact cells and in membranes from various tissues (*5*); binding can be altered or destroyed by treating cells with free or agarose-bound enzymes (*5*); the receptors are not detectable in intracellular membranes (*5*); adenylate cyclase activity in isolated membranes can be perturbed directly by physiological concentrations of insulin (*6*); and insulin receptors have now been extracted and purified from cell membranes (*7*). Although these studies are consistent with and further expand the observations made with insulin-agarose, it is very misleading to suggest (as Katzen and Vlahakes do in their first paragraph) that any such studies are based on a "basic premise" which is "justified" by the insulin-agarose studies. By analogy, the growing number of important studies concerning receptors for many peptide, cholinergic, and adrenergic hormones, and their localization to cytoplasmic membranes, are not based on studies with insoluble hormones.

A "unitary concept" of insulin action should in principle be viewed in the same way we evaluate the action of other hormones. In this respect the basic but still unanswered question is whether all of the metabolic effects of insulin can be explained on the basis of a single, unique, and fundamental biochemical event. This question is dependent not on interpretations of existing insulin-agarose studies, but on a better understanding of the detailed biochemical processes which follow the initial insulin-receptor interaction.

I take this opportunity to present additional evidence for the inherent activity of new and interesting insulin-agarose derivatives recently prepared in this laboratory. Insulin attached to agarose through very large macromolecular "arms," which consist of branched copolymers of poly-L-lysine-L-alanine can be demonstrated to be biologically active under conditions where no significant free insulin is released into the medium (Table 1). These derivatives may be especially useful because of the large distance which separates the insulin from the agarose backbone, and because of the great stability of the coupled insulin which results from the multipoint linkage of the copolymer to the agarose backbone.

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20 January 1973

Generation Time and Genomic Evolution in Primates

It is widely accepted among those who study evolution at the molecular level that time is a major factor determining the degree of sequence difference among the homologous macromolecules of different species. Whether done on the globins (*1*), cytochromes c (*2*), fibrinopeptides (*3*), albumins (*4–7*), lysozymes (*8*), or DNA's (*9*, *10*), such studies have generally shown a strong correlation between the degree of sequence difference and the time that has elapsed since the two species being compared last shared a common ancestor. Substantial disagreement persists, though, on the question of whether the time factor should be measured in terms of astronomical time or generation length (*9–12*). These conflicting hypotheses have been subjected to direct test, and we here review evidence developed in such tests which strongly supports the astronomical time hypothesis.

It is important at the outset to make clear the distinction between measuring absolute and relative rates of macromolecular evolution. We believe that the failure to make that distinction has led others (*9–12*) unwittingly to favor the generation time hypothesis. To measure absolute rates of macromolecular evolution requires known times of divergence between living species and is thus completely dependent on a detailed and properly interpreted fossil record. The method is illustrated by the following example. If it is known that the albumins of two species A and B differ in sequence by x amino acid replacements and that the A and B lineages diverged t million years ago, the average rate of albumin evolution in this case is x/t amino acid replacements per million years of separation. Although t is sometimes known precisely, this will not be true for most absolute rate measurements. It is relatively easy

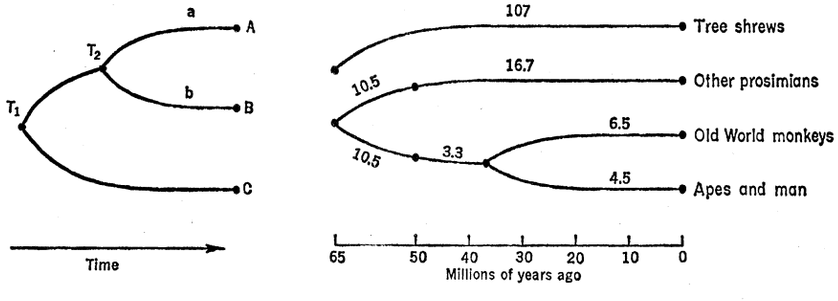

Fig. 1 (left). Phylogenetic relationships among three hypothetical living species, A, B, and C. The most recent common ancestor of A and B lived at time T_2, and the most recent common ancestor of all three species lived at time T_1; a and b are the amounts of sequence change (measured in amino acid replacements, immunological distance units, or nucleotide replacements) along the A and B lineages from T_2 until the present. The experimental A-B difference is assumed to be $a + b$; a and b can then be calculated if the A-C and B-C distances are known (*5*). Fig. 2 (right). Number of generations ($\times 10^6$) along various primate lineages according to the model of Lovejoy *et al.* (*12*). The divergence times are those used in (*12*); thus, we do not denote a specific divergence time for the tree shrews. For our calculations we have used the relationship between generations per year [$X(t)$] and time in millions of years (t) given in (*12*). However, one of their equations, $X_2(t) = 0.216t^{-0.181}$, is incorrect and should read $X_2(t) = 0.358t^{-0.181}$; we have used the latter in our calculations.