The Insulin Immunoassay After 50 Years A Reassessment

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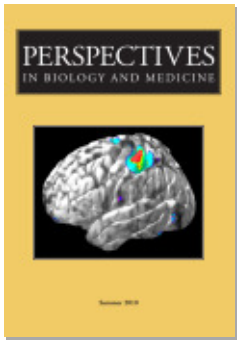

The Insulin Immunoassay After 50 Years

a reassessment

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ABSTRACT In 1960 Berson and Yalow published a method for the radioimmunoassay (RIA) of plasma insulin based on the concept that the extent to which unlabeled insulin displaces labeled insulin from anti-insulin antibody is proportional to the concentration of unlabeled insulin. The RIA for insulin has greatly increased knowledge of the physiology of glucose homeostasis and of the diverse causes of diabetes mellitus. Beyond this, the insight on which the RIA—or, more broadly, the competitive protein-binding assay—is based has provided the means to measure nanomolar or picomolar concentrations of a vast array of compounds in plasma and tissues. Directly or indirectly, the RIA has profoundly affected every branch of medicine. This essay reviews the ideas that were current in the medical research community when Berson and Yalow began their work and the observations and reasoning process that led them to their seminal discovery.

In 1960 Berson and Yalow published a method for the measurement of plasma insulin by radioimmunoassay (RIA). The insight that enabled them to conceptualize the RIA—that the extent to which unlabeled insulin displaces labeled insulin from anti-insulin antibody is proportional to the concentration of unlabeled insulin—had been described in a paper published four years earlier (Berson et al. 1956). That insight and the experimental ingenuity required to convert it to a procedure for the precise measurement of plasma insulin and other pep-

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tide hormones constitute one of the outstanding achievements of 20th-century biomedical science, comparable in importance, arguably, to the formulation of the tricarboxylic acid cycle, the elucidation of the structure of DNA, and the discovery of cyclic AMP.

The RIA for insulin has greatly increased knowledge of the mechanisms maintaining glucose homeostasis and deepened understanding of the pathogenesis of the different types of diabetes. However, its significance extends far beyond this achievement. RIAs and other competitive protein-binding assays have facilitated development of powerful analytic techniques, techniques that permit accurate measurement of nanomolar or picomolar concentrations of a vast array of compounds in plasma and tissues: hormones; vitamins; enzymes; antibiotics, cardiac glycosides, and many other drugs; DNA; RNA; tumor antigens; intrinsic factor; rheumatoid factor—indeed, in Yalow's (1992b) words, "virtually any substance of biologic interest" (p. 5). Directly or indirectly, the RIA has profoundly affected every branch of medicine.

This essay reviews some of the ideas and observations that shaped the intellectual climate in which Berson and Yalow began their studies of plasma insulin, summarizes these studies and Berson and Yalow's analysis of the technical problems that can complicate the validation of RIAs, and concludes with a few comments on the insulin RIA as a stimulus to the development of other hormonal assays, particularly during the decade following its publication.

In 1921 and 1922, the isolation of insulin by Banting, Best, McLeod, and Collip and the demonstration of its efficacy in the treatment of insulinopenic diabetes led, understandably, to the conclusion that diabetes was caused exclusively by lack of insulin. More than a decade passed before serious consideration was given to the possibility that diabetes might in some instances result from insulin resistance rather than insulin deficiency. In 1936, Himsworth presented experimental data supporting the conclusion "that two different types of disease can be distinguished as causing the symptom complex of diabetes mellitus. One, the insulin-sensitive type, appears to be caused by deficiency of insulin; the other, the insulin-insensitive type, is apparently due not to lack of insulin, but to lack of an unknown factor which sensitizes the body to insulin" (p. 130). In a prescient comment, he wrote: "As diabetes becomes more frequent with increasing age it would appear probable—and my experience so far supports this deduction—that the commonest type of diabetes mellitus will eventually prove to be that which is not essentially due to insulin deficiency" (p. 128). In the Goulstonian Lectures delivered three years later, Himsworth (1939) entertained the possibility of yet a third type of diabetes: "Diabetes mellitus is a disease in which the essential lesion is a diminished ability of the tissues to utilize glucose . . . referable either to deficiency of insulin or to insensitivity to insulin, although it is possible that both factors may operate simultaneously" (p. 175). If, as Himsworth postulated, there were several types of diabetes, one induced by insulin deficiency, a second by insulin resistance, and, perhaps, a third by a combination of these defects, then it

became a matter of considerable research interest and clinical relevance to correlate insulin requirements in diabetic patients with plasma insulin and glucose levels. This could not be done without a reliable assay for plasma insulin.

Starting in the 1940s, partly in response, perhaps, to Himsworth's analysis, protocols for the bioassay of insulin began to appear in the medical literature. The earliest assays measured the fall in blood sugar in rats in response to injection of a range of known concentrations of purified commercial or "standard" insulin. When an unknown sample of human plasma was administered to a rat from the same brood, its insulin concentration was assumed to be identical to that in the dilution of standard that caused an equivalent reduction in blood sugar. These studies revealed an extremely poor correlation between measured blood sugar levels and known or calculated insulin levels. This was true even when the test animals were first rendered diabetic by treatment with alloxan and then adrenalectomized or hypophysectomized in order to make them exquisitely sensitive to insulin (Anderson, Lindner, and Sutton 1947; Bornstein and Lawrence 1951). "Whole animal" insulin assays were in time replaced by in vitro bioassays, in which dose-response curves were constructed relating known insulin concentrations in incubation media to specific metabolic effects in isolated tissues, such as the rate of glucose uptake or glycogen synthesis in rat hemidiaphragm or the rate of glucose oxidation in rat epididymal fat pad (Groen et al. 1952; Martin, Renold, and Dagenais 1958; Vallance-Owen and Hurlock 1954).

These in vitro bioassays for plasma insulin yielded highly variable results from one assay to the next and were therefore of little value to researchers or clinical diabetologists. Several other factors contributed to loss of confidence in insulin bioassays: growing doubt that they were specific for insulin; the virtual impossibility of preparing for each assay tissue samples—whether diaphragm, epididymal fat pad, or liver—with reproducible responses to a range of insulin concentrations; and an increasing body of evidence that plasma contained both noninsulin substances with insulin-like effects and insulin antagonists, and that insulin was degraded in liver and other organs.

The evidence for circulating insulin-like materials as well as insulin antagonists was highly suggestive, if not conclusive. With regard to the former, Leonards (1959) described a substance in normal human fasting serum that, like insulin, stimulated glucose oxidation and triglyceride synthesis in fat tissue but that, unlike insulin, could not be extracted from plasma into acid-alcohol. While serum from guinea pigs immunized against insulin suppressed insulin action in fat tissue, it had no effect on Leonards's insulin-like substance. Froesch et al. (1963) distinguished nonsuppressible insulin-like activity or NSILA (activity unaffected by anti-insulin serum) from suppressible insulin-like activity (activity abolished by anti-insulin serum and thus attributable exclusively to insulin). To add to these mounting complexities, Antoniades (1961) described two forms of plasma insulin, one with diminished activity that was bound to plasma proteins, the other unbound and fully active.

Insulin antagonists had been identified in the sera of ketotic and nonketotic diabetics and of normal subjects. How, or whether, these antagonists were related to each other was uncertain (Field, Tietze, and Stetten 1957; Steinke, Taylor, and Renold 1961; Vallance-Owen 1960). Moreover, Mirsky (1952) had identified insulinase, an insulin-degrading enzyme that was widely distributed in human and animal tissues, and had proposed that increased insulinase activity might catabolize insulin at a rate sufficient to cause diabetes.

By the mid-1950s, then, it was obvious that a fundamentally different approach to the measurement of plasma insulin was needed. Development of the insulin immunoassay was dependent on the antigenicity of insulin. In fact, when Berson and Yalow established their laboratory there was already an abundance of data best explained by the hypothesis that insulin could induce antibody production. Nonetheless, some authorities rejected this idea because it was at variance with the dogma that antigens had to be of high molecular weight. For that reason, it was argued, insulin, a comparatively small peptide, could not act as an antigen. What, then, was the evidence for and against the existence of anti-insulin antibodies?

Not long after their introduction for the treatment of diabetes, both beef and pork insulins were found to cause two types of effects that could be most simply understood as immunologic responses to commercial preparations of the hormone (or to accompanying impurities): first, wheal and erythema reactions at and sometimes remote from injection sites or, far less commonly, systemic allergic reactions (bronchoconstriction, hypotension, etc.); secondly, insulin resistance (Lowell 1944; Wright 1960). A number of authors suggested that insulin-mediated allergy and resistance to insulin therapy were due, respectively, to reaginic or skin-sensitizing antibodies and to antibodies located in the gamma globulin fraction of plasma proteins.

In 1938, Banting and colleagues reported the case of a nondiabetic, schizophrenic patient who, during the course of insulin shock therapy, became so resistant to the hormone that after 59 treatments an injection of 1,000 units of insulin reduced the blood sugar level by a mere 35 mg/dl (from 115 mg/dl to 80 mg/dl). The patient's serum contained a nondialyzable factor that precipitated with the globulin fraction and protected fasting mice from insulin-induced hypoglycemic convulsions (Banting, Franks, and Gairns 1938). Lowell (1944) provided one of the earliest accounts of a patient with diabetes who manifested both insulin allergy (generalized urticaria) and resistance. In a subsequent paper, he described another patient with diabetes and schizophrenia who became allergic and resistant to insulin during insulin shock therapy. The patient's serum protected fasting mice from insulin-induced hypoglycemia (Lowell 1947).

In 1949, Lowell and Franklin reported experiments in which repeated subcutaneous injections of beef/pork insulin were given to rabbits. Four of 24 animals became resistant to this insulin mixture. The resistance was species-specific: in the single rabbit described in detail, normoglycemia persisted and a substantial

decrease in blood sugar level followed the injection of human insulin (demonstrating sensitivity both to endogenous and human insulins).

In 1952, De Filippis and Iannacone described a 45-year-old woman with diabetes who remained hyperglycemic despite an insulin dosage of 460 units per day. The gamma globulin fraction of her serum prevented insulin-induced hypoglycemia in 18 of 25 starved rats.

In a study published in 1955, Moloney and Coval induced anaphylactic shock in guinea pigs previously sensitized to pig or ox insulin by the intravenous or intracardiac injection of pig, ox, or sheep insulin. Serum pooled from guinea pigs sensitized to pig insulin prevented pig insulin–induced hypoglycemic convulsions in mice, caused hyperglycemia when administered alone, and blocked the hypoglycemic effects of subsequent injections of pig, but not of guinea pig, insulin.

By 1955, Sanger and his colleagues had determined the structure of ox insulin and had concluded that its molecular weight was approximately 6,000 (Ryle et al. 1955). As noted above, a central immunologic precept was that compounds with molecular weights in excess of 10,000 tended to be effective antigens, provided that they were "foreign" to the animals exposed to them. Peptides or polysaccharides with molecular weights of 5,000 or less usually proved to be poor antigens, unless they were conjugated with proteins or injected with Freund's complete adjuvant, a suspension of killed mycobacteria in water and mineral oil that nonspecifically enhances antigenicity by provoking an inflammatory reaction (Kabat and Bezer 1958; Sela 1966). Thus, despite the many experimental and clinical observations cited above, all compatible with the concept that insulin can act as an antigen when donor and recipient insulins possess chemically dissimilar epitopes, the low molecular weight of the hormone persuaded more doctrinaire critics that it was probably incapable of inducing antibody production.

In 1956, Arquilla and Stavistsky published an insulin assay based on the antigenic properties of the hormone. Beef insulin was coupled to sheep or rabbit erythrocytes, using bis-diazobenzidine as the coupling agent. Addition of insulin antiserum (prepared by giving rabbits multiple injections of beef insulin) and complement to the insulin-coupled erythrocytes caused them to hemolyze. The amount of hemoglobin thus released was proportional to the degree of hemolysis and was measured spectrophotometrically. When beef insulin was included in the insulin-coupled erythrocyte/antiserum/complement mixture, it bound the beef insulin antibodies, diverting them from the erythrocytes and thus decreasing hemolysis. Since there was a quantitative relationship between the concentration of added insulin and the extent to which hemolysis was suppressed, it seemed possible that this procedure could be used to assay insulin. Arquilla and Stavitsky (1956) were able to measure micromolar concentrations of insulin, concentrations 100- to 1,000-fold greater than clinically relevant concentrations. It was apparent, therefore, that despite its ingenuity, this assay could have no useful clinical or research application.

In her 1977 Nobel lecture, Yalow (1992b) recalled that the development of the RIA was an unanticipated consequence of the effort to test Mirsky's hypothesis concerning the etiology of diabetes: "Radioimmunoassay came into being not by directed design but more as a fall-out from our investigations into what might be considered an unrelated study. Dr. I. Arthur Mirsky had hypothesized that maturity-onset diabetes might not be due to a deficiency of insulin secretion but rather to abnormally rapid degradation of insulin by hepatic insulinase" (p. 5).

Yalow, whose training was in nuclear physics, earned a Ph.D. from the University of Illinois-Urbana in 1945. Her doctoral dissertation was an inquiry into the mechanism by which tellurium (121/52 Te) decays to antimony (121/51 Sb) (Yalow 1945). In 1946, Yalow began to work in the laboratory of Dr. Edith Quimby, a radiation biologist at the College of Physicians and Surgeons, Columbia University. It is reasonable to assume that her association with Dr. Quimby encouraged Yalow to believe that radioisotopes could be employed to great advantage in studies of human disease. In 1950, Yalow obtained a full time position as Physicist and Assistant Chief of the Radioisotope Service at the Bronx Veterans Administration Hospital. A few months later, conscious of the need for a clinician to assist her in selecting problems that could be productively investigated with radioisotopes, she began a collaboration with Dr. Solomon Berson, an intellectually gifted young physician and polymath. This collaboration continued until Berson's death in 1972 (Friedman 2002; Straus 1999). Early studies in euthyroid, hyperthyroid, and hypothyroid subjects utilized I^{131} to estimate rates of synthesis, degradation, and clearance of protein-bound iodine (essentially equivalent to thyroid hormone) and the extrathyroidal volume of distribution of organic iodine (Berson and Yalow 1954). In other work, plasma volume, expressed as the volume of distribution of I^{131}-labeled human serum albumin, was measured in patients with congestive heart failure before and after treatment (Schreiber et al. 1953).

In the radionuclide I^{131}, Berson and Yalow had an excellent tool with which to test Mirsky's hypothesis that diabetes may result from an excessively rapid rate of insulin degradation. If Mirsky was correct, intravenously administered I^{131}-labeled insulin should disappear from the plasma of diabetic subjects more rapidly than from nondiabetic plasma. As it turned out, Mirsky was proved wrong. In their landmark paper published in the *Journal of Clinical Investigation* in 1956, Berson and Yalow wrote: "In subjects treated with insulin for months to years the disappearance of insulin-I^{131} from plasma is much slower than in subjects never treated with insulin or treated with insulin for less than two-three months. It has been shown that this persistence of relatively high concentrations of insulin-I^{131} in the plasma of insulin-treated subjects is due to binding of insulin-I^{131} by an acquired globulin which satisfies the criteria for antibody" (Berson et al. 1956, p. 189). The reviewers of the paper, who questioned the antigenicity of insulin and had rejected an earlier version of the work, continued to object to the unqualified designation of the "acquired globulin" as an antibody but

were somehow willing to accept the essentially equivalent statement that it exhibited properties that defined an antibody.

The 1956 paper was followed by kinetic studies of the rates of formation and dissociation of insulin-antibody complexes when I^{131}-labeled beef insulin and antisera from insulin-treated patients were incubated at different relative concentrations (Berson and Yalow 1958, 1959a). Scatchard plot analyses were carried out to determine the insulin binding capacity of human antiserum to beef/pork insulins incubated with beef, pork, sheep, and horse insulins (Berson and Yalow 1959b). In the 1958 paper cited above, the concentrations of beef insulin in rabbit plasma were measured at frequent intervals in nine rabbits following intravenous injections of beef insulin. In this assay, trace amounts of beef insulin I^{131} and human antiserum to beef insulin were added to rabbit plasma (Berson and Yalow 1958). These studies helped to clarify the pathogenesis and clinical expression of immunogenic insulin resistance.

In 1959, Yalow and Berson reported the immunoassay of fasting and post-glucose plasma insulin levels in two normal human subjects. In this communication, a preamble to the groundbreaking 1960 paper, beef insulin I^{131} and guinea pig antiserum to beef insulin were added to human plasma (Yalow and Berson 1959).

The crucial observation that led to the development of the RIA for insulin was that addition of increasing concentrations of unlabeled (standard) insulin to a fixed amount of I^{131}-labeled insulin progressively displaced the latter from its binding to a fixed amount of insulin antibody. This observation permitted construction of standard curves that could be used to measure the insulin concentration of any plasma sample. Guinea pig antiserum to beef insulin supplied the antibody, and the ratios of bound to free I^{131} beef insulin were plotted against known concentrations of unlabeled human insulin to generate a "standard curve." In order to minimize damage to the iodinated beef insulin, plasma was prepared in 1:10 or greater dilutions, and incubations were carried out at 4°C for four days (Yalow and Berson 1960). Although standard curves typically plotted the ratios of bound to free labeled hormone against increasing concentrations of unlabeled hormone, such curves could also be constructed by plotting labeled bound hormone, or its reciprocal, or labeled free hormone against unlabeled hormone (Berson and Yalow 1976).

In their 1960 *Journal of Clinical Investigation* paper, Berson and Yalow measured plasma glucose and insulin concentrations in nondiabetics and maturity-onset diabetics before and after an oral glucose load, and they found that the diabetic subjects exhibited both grossly impaired glucose tolerance and abnormally high post-glucose plasma insulin levels. This unanticipated result called for explanation, and their comments were perceptive: "To resolve the present finding of a higher than normal integrated insulin output in diabetics during the glucose tolerance test with sustained hyperglycemia in these patients, it must be concluded that the tissues of the maturity onset diabetic do not respond to . . . insulin as well as the tissues of the nondiabetic subject" (p. 1173). In revealing the fre-

quency with which maturity-onset diabetes is associated with insulin resistance, they confirmed the conclusion that Himsworth had reached in 1936.

Berson and Yalow analyzed the many technical problems that can complicate performance and interpretation of the insulin RIA: the presence of anti-insulin antibodies in insulin-treated subjects; incomplete separation of the bound and free fractions of labeled insulin; failure to prepare an insulin standard; and plasma insulin samples that are immunologically identical. They cautioned that a competitive protein-binding assay could not be validated without confirming the immunologic identity of the hormone standard and the hormone in the specimen of unknown plasma. This, in turn, required: (1) that the concentration of hormone in unknown plasma decrease linearly with dilution; and (2) that the curves relating bound-to-free ratios of labeled hormone to concentrations of unlabeled hormone in standard solutions and plasma unknowns be superimposable. Failure to achieve superimposition of these curves could be explained by derivation of standard and plasma hormone from different species, the presence of hormonal fragments, "immunologically related hormones of [the] same species," "heterogeneity of hormonal forms in standards or unknowns," or the effects of "nonspecific factors" (ionic environment, pH, heparin, radiation-induced damage to labeled hormone during the process of iodination) (Yalow and Berson 1971).

The final section of the 1960 paper discussed the paradox that serum insulin concentrations determined by bioassay usually greatly exceeded insulin concentrations determined by RIA in the same specimens. In attempting to account for this discrepancy, Berson and Yalow noted that Leonards's finding of NSILA in normal human fasting plasma "has raised a serious question as to what part of the insulin effect on fat tissue is due to insulin itself." They then suggested that the "much higher estimates of plasma insulin concentration" determined by bioassay may, indeed, have been attributable to the presence of noninsulin substances with insulin-like activity. Even if one assumed, they continued,

> that endogenous insulin is confined almost exclusively to the plasma . . . a fasting level as high as 4600 microunits/ml (as reported by Willebrands) . . . is still difficult to accept. Randle's values of 9000–22,000 microunits/ml in normal plasma 2.5 hours following glucose would mean a total of 27 to 66 units in plasma alone, neglecting insulin in the extravascular space, at a time when the blood sugar is usually at a normal level. However . . . Randle has indicated his conviction that this insulin-like activity is not due entirely to insulin alone. (p.1172; see also Randle 1954; Willebrands, Geld, and Groen 1958)

Between 1960 and 1970, Berson and Yalow published immunoassays for human growth hormone, ACTH, parathyroid hormone, and gastrin (Berson and Yalow 1976; Yalow and Berson 1976). Their work had immense impact. During the same decade, many investigators adapted the techniques of RIA to the measurement of other peptide hormones, nonpeptide hormones, and metabolic in-

termediates such as cAMP. Murphy and colleagues reported assays for plasma cortisol and corticosterone and for thyroxine, in which the unlabeled hormones displaced, respectively, cortisol-C^{14} from cortisol-binding globulin and thyroxine-I^{131} from thyroxine binding globulin (Murphy, Engelberg, and Pattee 1963; Murphy and Pattee 1964). Goodfriend, Levine, and Fasman (1964) developed assays for bradykinin and angiotensin, in which these peptides, each with a molecular weight of about 1,000, were rendered antigenic by conjugating them with rabbit serum albumin. In 1969, two assays for estradiol were published, both of which employed 17β-6,7-^{H3}estradiol as the radioligand. In one, antiserum was obtained from a ewe immunized with an estradiol-bovine serum albumin conjugate; in the other, labeled and unlabeled hormone competed for a uterine cytosolic binding protein (Abraham 1969; Korenman, Perrin, and McCallum 1969). Walton and Garren (1970) measured cAMP in various rat tissues by progressive displacement of c(H3)AMP from its binding to a protein associated with protein kinase A. Lefkowitz, Roth, and Pastan (1970) measured ACTH utilizing ACTH-I^{125} as the radioligand and murine adrenal cell surface receptors to combine with the labeled and unlabeled hormone, an innovative approach, it was said, in which measured concentrations of the hormone more reliably reflected its biological activity.

No account of the history of RIA can omit mention of Berson's tragic death. Had he lived, he would surely have shared the Nobel Prize with Yalow. In her 1977 Nobel lecture, Yalow (1992b) spoke of his indispensable role in their collaboration: "From 1950 until his untimely death in 1972 Dr. Solomon Berson was joined with me in this scientific adventure and together we gave birth to and nurtured in its infancy radioimmunoassay, a powerful tool for determination of virtually any substance of biologic interest. Would that he were here to share this moment" (pp. 4–5). In the autobiographical vignette that accompanied the Nobel lecture, she added:

> Sol's leaving the laboratory in 1968 to assume the chairmanship of the Department of Medicine at the Mount Sinai School of Medicine and his premature death four years later were a great loss to investigative medicine. At my request, the laboratory which we shared has been designated the Solomon A. Berson Research Laboratory so that his name will continue to be on my papers as long as I publish and so that his contributions to our Service will be memorialized. (Yalow 1992a, p. 22)

Berson and Yalow did not patent their methods. Indeed, they made them readily available to colleagues, so it might be argued that unrestricted access to these methods actually accelerated the application of RIA to the measurement of hormones other than insulin. However, their evaluations of the work of other investigators were, perhaps, inordinately influential in the medical research community, and it has been suggested that their dismissive attitude toward some contemporaries impeded important advances in scientific knowledge. One assertion

is that they decried the notion of NSILA and, in so doing, delayed the identification of insulin-like growth factors (Kahn and Roth 2004). It should be noted, however, that their published comments, while indicating uncertainty about the physiologic role of NSILA, were in no sense derisive.

Berson and Yalow have also been criticized for rejecting Vallance-Owen's report of an albumin-associated insulin antagonist (probably albumin-bound fatty acids); for challenging the concept, now unequivocally established, of insulin autoimmunity; and for disputing studies, later confirmed, which concluded that peptide hormones bind to specific cell surface receptors in target tissues (Kahn and Roth 2004). Even if one acknowledges merit in these criticisms, they do not diminish the enormous significance of RIA, a technological breakthrough that, more than any other, has helped to transform biomedical science into a quantitative discipline.

In remarks preceding Yalow's Nobel lecture, Professor Luft of the Karolinska Institute emphasized the wide-ranging impact of Yalow and Berson's work:

> The Yalow-Berson method which makes it possible to determine the exact amounts of all hormones present represented a real revolution in the field of hormonal research. A field where one refers to the time period before Yalow and the new epoch which began with her achievement. Her methodology, and the modifications thereof, subsequently made their triumphant journey far beyond her own field of research, reaching into vast territories of biology and medicine. It has been said that Yalow changed the life of a multitude of researchers within these fields. Rarely have so many had so few to thank for so much. (Luft 1992, p. 2)

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