THE PROLONGATION OF INSULIN ACTION

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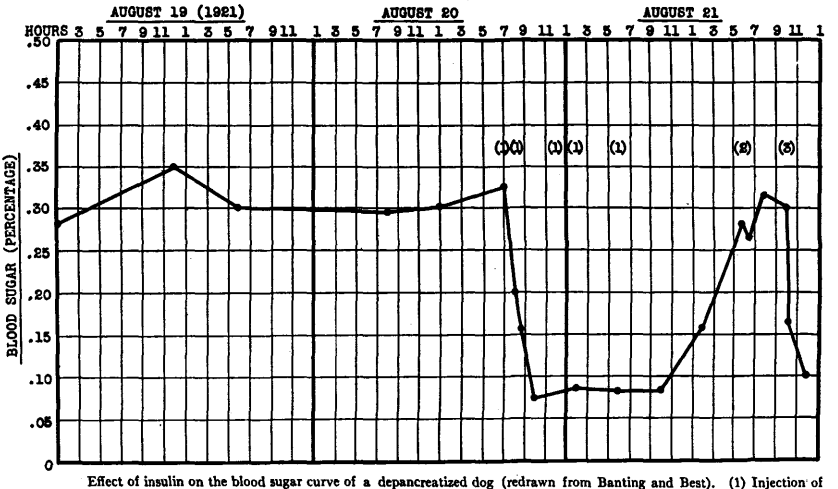
As an introduction to the consideration of the different forms of insulin, which will be discussed later, a few remarks may be made about the anti-diabetic material which was available in 1921. The administration of an effective dose of this material caused a fairly prompt fall of the blood sugar to a low level. The action was maintained for an appreciable length of time (Fig. 1). It is readily apparent to physiologists, who have worked with various preparations of insulin, that the crude material available 16 years ago had a much more prolonged action than the more highly purified preparations. While there is considerable difficulty in making an accurate comparison of materials used in 1922 and, let us say, in 1930, many clinicians also agree that the crude preparations exerted a much more prolonged effect than the solutions of the purified insulin. The removal of impurities decreased the incidence of local reactions at the site of the injection but did not improve insulin as a specific therapeutic agent.

Insulin was available in a very satisfactory state of purity before the substance was isolated in crystalline form. The preparation of the crystals was, from the chemical viewpoint, an extremely important landmark in the history of the development of the subject. This was accomplished by Abel, Geiling, Rouiller, Bell and Wintersteiner in 1927. The crystals occur in several forms and are to some extent interconvertible. The recent work of my colleague, D. A. Scott (1935, 1936), on the rôle of zinc in the crystallization of insulin has placed the preparation of these crystals on a more scientific basis. It is now a relatively easy matter to obtain large amounts and a fair yield (75 per cent or higher) from suitable amorphous material. The crystalline material was termed by Scott "zinc insulinate." Other metals may also be used in the preparation of the crystals and these combine with insulin in amounts which are proportional to their atomic weight. The average ash content of each insulin salt is proportional to the atomic weight of the metal it contains. This indicates that the crystals contain the metals as chemically combined constituents and not as impurities. Scott

and Fisher (1935) have found that if the molecular weight of insulin be taken as 20,000 each formula weight of insulin combines with 1.4 formula weights of cadmium, with 1.5 formula weights of cobalt and 1.6 formula weights of zinc. If the molecular weight of insulin should be 40,000 one formula weight would therefore combine with approximately three formula weights of the metals. Svedberg, using his ultracentrifuge technique, has suggested 35,100 for the molecular weight of insulin and Crowfoot, on the basis of X-ray studies, a value of 37,200.



Effect of insulin on the blood sugar curve of a depancreatized dog (redrawn from Banting and Best). (1) Injection of extract of degenerated pancreas; (2) extract after incubation with pancreatic juice; (3) extract incubated without pancreatic juice. Blood sugars by Myers-Bailey modification of Lewis-Benedict method.

FIGURE 1

The chemical structure of these crystals of insulin has attracted a great deal of attention. This is not surprising since there are relatively few crystalline proteins which can be so readily assayed. An appropriate idea of the potency can be obtained when only a few milligrams of the material are available. There is an abundance of evidence that the crystalline material is protein in nature. Eight amino acids have also been isolated—cystine, tyrosine, arginine, histidine, lysine, leucine, glutamic acid and phenylalanine. Others are quite likely present. In the search for the active groupings in the molecule attention has been directed at the NH_2 group and the disulphide linkages. Destruction of the amino group irreversibly inactivates insulin. When the disulphide grouping is reduced to the

sulphydryl the specific activity is lost—some investigators believe irreversibly while others think that a partial inactivation which can be reversed may be produced. An insulin-plastein may be formed when the products of peptic digestion of insulin are allowed to interact under certain conditions but this, as Scott considered likely before he performed the experiment, possessed no anti-diabetic activity. The complexity of the insulin molecule makes the possibility of synthesis remote.

The international yardstick for insulin is the new crystalline standard. Fifty grams of crystals were prepared in the Connaught Laboratories by Dr. Scott and presented to the Health Organization of the League of Nations by the Insulin Committee of the University of Toronto. The potency of the new standard was compared to that of the old amorphous preparation in a number of laboratories. The results were submitted to representatives of the National Institute for Medical Research, the University of Copenhagen and the University of Toronto (represented by Sir Henry Dale, Professor August Krogh and the writer). A recommendation was made that a value of 22 units per mg. be assigned to this new standard. It was felt that this would not change the strength of the unit. This recommendation was accepted by the Health Organization of the League of Nations (1936). The unit of insulin is, therefore, defined as the activity contained in $\frac{1}{22}$ of a mg. (0.045 mg.) of the new International Standard.

IMPROVING INSULIN AS A THERAPEUTIC AGENT

The advances recently made in this field have prompted me to discuss it in some detail.

Various efforts were made in 1922 and 1923 to find a means of administering insulin by the enteral route. Very little success was secured, and if we now take all the reports on this problem into account, the situation is approximately as follows: Insulin is destroyed by the enzymes of the stomach and small intestine. Small amounts of insulin may be absorbed from the small intestine, particularly in young animals when very large doses are given. The rate of absorption is not predictable. There is no appreciable absorption from the large intestine. The situation with respect to the absorption of insulin from other mucous membranes is not more promising. An anti-diabetic effect can be observed when an insulin solution is inhaled, but results of practical value have not been secured. A very little insulin

may be absorbed through the skin when vigorously rubbed in with a lanoline base.

It is well known that the anti-diabetic hormone is rapidly absorbed when given intraperitoneally, intramuscularly or subcutaneously. The rate of action of purified insulin is only slightly less when given subcutaneously than when administered into a vein, and intramuscular injection does not appreciably delay absorption. We can, therefore, restrict our discussion very largely to the question of modification of the rate of action of insulin which is administered subcutaneously.

As stated above there is an abundance of evidence to show that crude insulin exerts a more prolonged effect than the purified material. It is also well established that a given amount of insulin administered in several small doses exerts a greater effect than when the same amount is administered in large doses. This effect is due, probably, to the relatively large amount of insulin excreted or destroyed when the large doses are employed. These facts have stimulated investigators to attempt to secure the prolongation of the absorption of insulin from the subcutaneous tissue spaces. The first report of an attempt to do this was that of Burgess, Campbell, Osman, Payne and Poulton (1923), who used insulin in gum arabic solutions. These investigators secured a definite prolongation of insulin action in diabetic patients and in experimental animals. Similar results were obtained by de Jongh and Laqueur (1925). Leyton (1929) convinced himself of the efficiency of insulin solutions mixed with certain oils. He was able to give larger doses, and apparently expected the use of oily suspensions of insulin to become general. Surányi and Szalai (1930) obtained a prolonged action and less frequent hypoglycaemic reactions when insulin was mixed with lecithin emulsions. Skouge and Schrumpf (1932) confirmed the lecithin results and found, as might perhaps be expected, that no extension of the insulin effect was obtained when the mixtures were given intravenously. They found it possible to administer subcutaneously large doses of insulin in lecithin without the production of hypoglycaemic reactions. Several groups of investigators—Wermer and Monguió (1933) and Clausen (1934)—attempted to prolong the action of insulin by administering pituitrin or epinephrin with the insulin solution. Little success attended these efforts.

Apparently the first attempt to modify the action of insulin by the use of metals was that of Bertrand and Mâcheboeuf

(1926). These investigators reported a very definite extension of insulin action when cobalt and nickel were added to the insulin solutions. This report led to the development of a controversy, and the findings have not been generally confirmed. We must not lose sight of the fact, however, that Bertrand and his collaborator were the first to suggest the use of a metal in this connection. The blood sugar curves which Maxwell and Bischoff (1935) have published appear to indicate that basic ferric chloride definitely slows the absorption of insulin. These investigators have used other metals including zinc in their studies of other hormones.

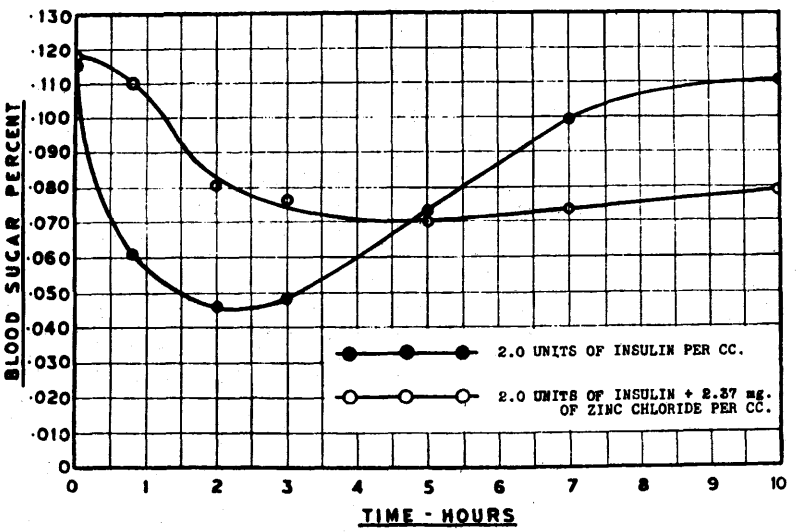


FIGURE 2. (From Scott and Fisher.)

Scott and Fisher were led to a study of the action of zinc salts on the rate of absorption of insulin by the findings referred to above, that zinc, or some closely related metal, was an essential component of crystalline insulin. The addition of the appropriate amount of zinc to insulin solution definitely delays the absorption of insulin. It is possible that the zinc forms a compound between the insulin and some component of the tissues, or that the zinc solution delays the absorption of the insulin by an astringent action or by some other mechanism. Scott and Fisher have reported very definite findings in rabbits (Fig. 2), and Dr. Kerr, working under my general direction, has confirmed their findings in the dog (Fig. 3). Dr. Walter Camp-

bell in Toronto and Dr. Rabinowitch in Montreal have recently used zinc insulin in diabetic patients. A prolonged insulin effect has been observed.

PROTAMINE INSULIN

This brings us to a consideration of protamine insulin which has been used with very considerable success for more than two years by Hagedorn (1936) and his colleagues in Copenhagen. The chemical background of this ingenious procedure for delaying the absorption of insulin is most interesting and I will now digress for a moment to discuss it.

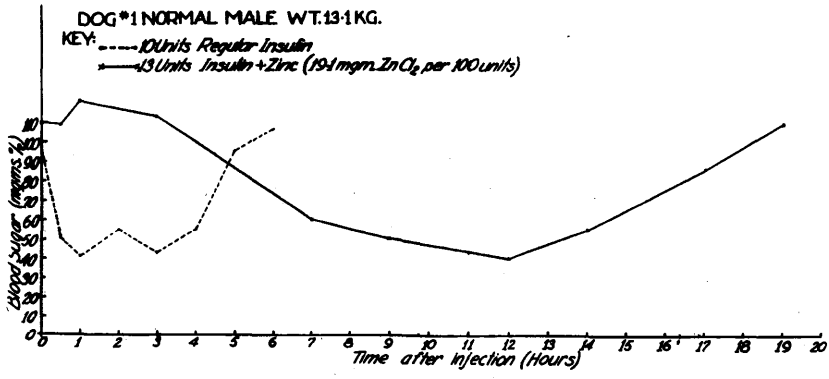


FIGURE 3. (From Kerr and Best.)

In 1868 Friedrich Miescher began an investigation of the chemistry of cell nuclei. This work, conducted in Hoppe-Seyler's laboratory, showed that proteins of acidic character containing phosphoric acid were present in nuclei of pus cells. Later he found these nuclions of nucleic acids in combination with basic substances in the sperm of salmon. He named the basic substances protamines. Some sixteen years later Kossel detected another type of basic substance in combination with nucleic acid. These were later shown to be similar to but somewhat more complex than the protamines. They are now called the histones. Kossel also isolated a protamine from the sperm of the sturgeon, which resembled but was not identical with the base from salmon sperm. Protamine was adopted as the general name for these bases while each separate one was called after the family name of the fish from which it was secured—thus sturine (sturgeon) etc. This protamine contains arginine, lysine

and histidine, and is one of the triprotamines. Sturine also contains alanine and leucine. Clupeine obtained from herring sperm is a monoprotamine and contains arginine as the only basic constituent. Alanine, serine, an aminovaleric acid, and proline were also present. Salmine is also a monoprotamine and contains serine, aminovaleric acid and proline as well as arginine. Hagedorn and his colleagues have prepared a new protamine—probably of the mono- variety—from the sperm of Salmo irideus (salmiridine) and they have used this in their clinical experiments, with protamine insulin.

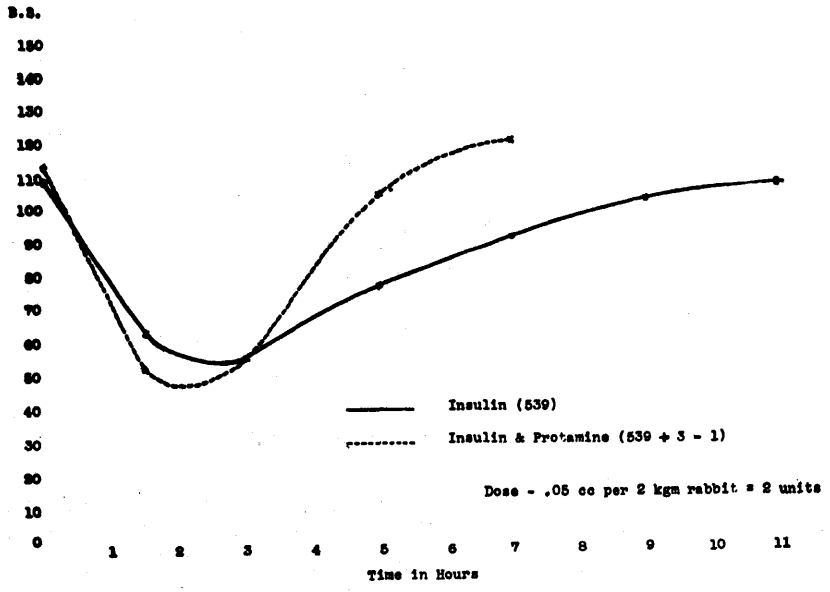


FIGURE 4. (From Scott and Fisher.)

Proteins are known to combine with the so-called protamines. The compounds are soluble at a certain hydrogen ion concentration, but insoluble at others. Hagedorn and his collaborators have very ingeniously taken advantage of this fact and have demonstrated beyond doubt that the compound of insulin and protamine is a very effective therapeutic agent in certain cases. A clear solution of protamine is added to insulin. The protamine is dissolved in a buffer solution of such a composition that the hydrogen ion concentration of the insulin-protamine mixture is approximately pH 7.2. At this pH the insulin-

protamine compound is insoluble and forms a flocculent precipitate. If the vial containing the mixture is shaken and the suspension administered, the insulin is slowly liberated in the tissues and a prolonged anti-diabetic effect is exerted. That this is due to slow absorption has been demonstrated directly by Beecher and Krogh (1936) who watched the rate of disappearance of regular and protamine insulin from the tissue spaces. The effect of protamine insulin on rabbits or dogs is easily demonstrated (Figs. 4 and 5).

It has now been agreed by most diabetic specialists that protamine insulin has certain advantages over regular insulin in many cases of diabetes. The advantages are as follows:

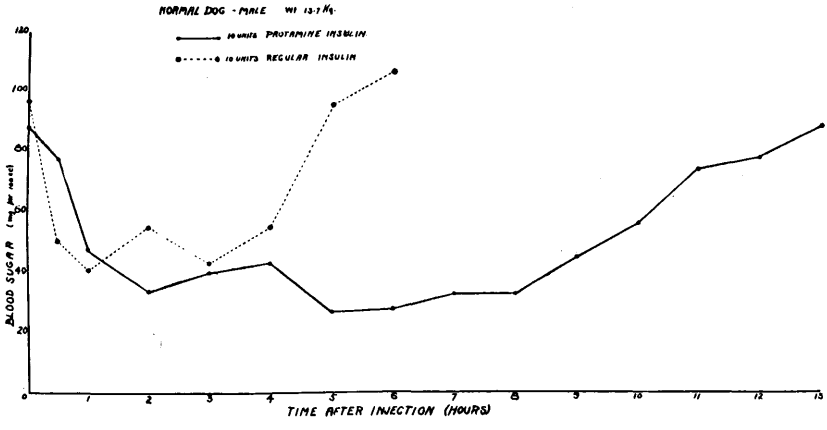


FIGURE 5. (From Kerr and Best.)

(1) The duration of the anti-diabetic effect is longer with protamine insulin than with the regular product. The number of injections required is, therefore, reduced. (2) The blood sugar curve is more regular and does not show the high peaks and marked depressions which are often observed with the regular material. (3) The ketosis is reduced when protamine insulin is used. (4) There are fewer hypoglycaemic reactions. (5) The patients feel better.

ZINC AND PROTAMINE INSULIN

When the protamine solution is mixed with the insulin and the insoluble precipitate formed, the resulting suspension of protamine insulin is not stable indefinitely. It has been found in the Connaught Laboratories and in the Insulin Committee's

testing department that in some cases after a few days a portion of the material sticks to the sides of the glass vial (Fig. 6) and that it is impossible to secure samples of uniform potency. It has been found by Scott and Fisher that this situation is corrected by the addition of a small amount of zinc to the protamine insulin mixture. The zinc in some way stabilizes the suspension and prevents it sticking to the glass vial. If this were the only effect of zinc it would be a very significant contribution but there are grounds for belief that zinc plays a rôle in the formation of a protamine insulin compound. Thus Scott and Fisher showed (1936) that when preparations of insulin and protamine from which inorganic material has been in large part removed were mixed a prolonged anti-diabetic effect was not secured. If, however, a small amount of zinc was present in this mixture, a slow absorption of insulin and a prolonged action were consistently produced. These results on rabbits have been confirmed by Kerr and Best (1937) on dogs. (Fig. 7). This compound of protamine and insulin to which zinc has been added has been named by the Toronto group "protamine zinc insulin." The use of zinc has made it possible to distribute the preparation in one vial. The suspension is stable for at least six months and perhaps for much longer periods of time. The protamine used in the Connaught Laboratories has been prepared from the mature testes of several species of British Columbia salmon, namely, spring salmon (Oncorhynchus tschawytscha), cohoe salmon (Oncorhyncus kisutch) and the steelhead (Salmo gairdneri). These products are quite as satisfactory as those secured from rainbow trout (Salmo irideus) and are readily available in large quantities.

"FREE INSULIN" IN THE PROTAMINE ZINC INSULIN MIXTURE

Some clinicians have raised objections to the use of protamine insulin and protamine zinc insulin on the grounds that they do not exert their effects with sufficient rapidity. This has been overcome by the administration of a small amount of regular insulin with the protamine zinc insulin mixture. Quite recently a preparation, made by Fisher and Scott, which contains in addition to protamine zinc insulin a certain proportion of "free insulin," has been tested by Kerr and McCutcheon under my general direction in the Department of Physiology. It has been found that a rapid effect comparable to that obtained with regular insulin is secured and, further, that a prolonged

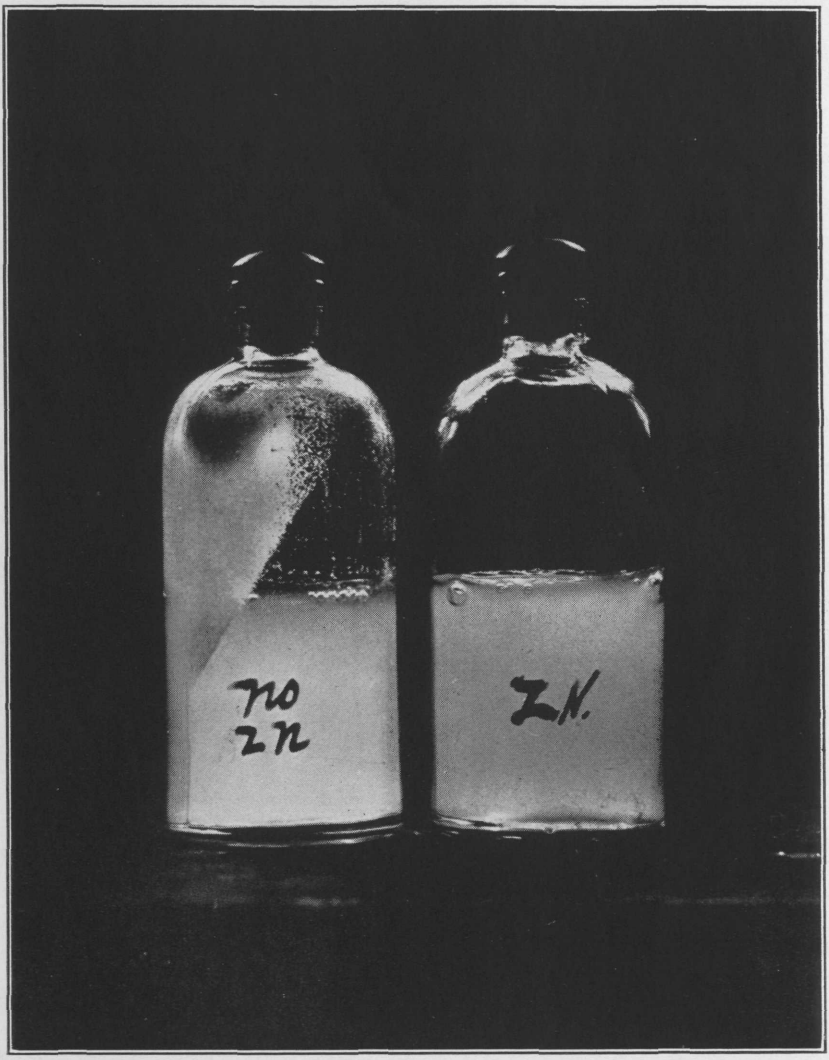
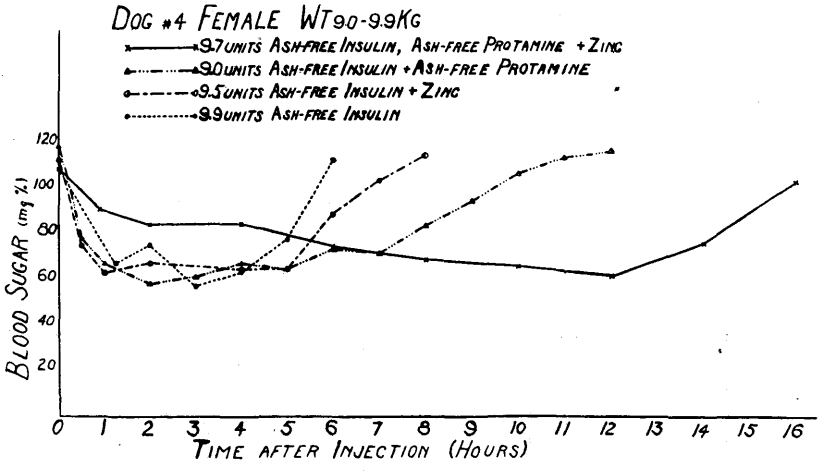


FIGURE 6

action is also produced (Fig. 8). There are some difficulties to be solved before a preparation of this kind suitable for clinical use can be made but it seems probable that a mixture which



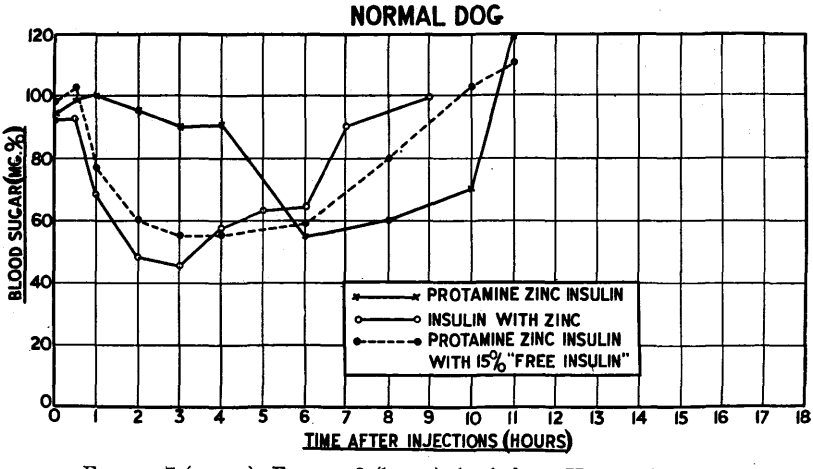


FIGURE 7 (upper); FIGURE 8 (lower), both from Kerr and Best.

will produce a rapid anti-diabetic effect and also a prolonged one, will, in due course, be made available. Clinical trial of this mixture is now being made.

PROTAMINE INSULIN IN DEPANCREATIZED DOGS

While the use of protamine insulin in diabetic patients had been investigated in some detail before these experiments on dogs were initiated considerable interest is attached to the findings in a completely depancreatized animal. Dr. Kerr has found it possible to keep several of the members of our colony of diabetic dogs in an excellent state of health and on a very liberal diet with one dose of protamine or protamine zinc insulin daily—20 units approximately for a 10 kilogram dog. The animals excrete only small amounts of sugar of the same order that are observed with two doses of regular insulin. When the daily

TABLE I

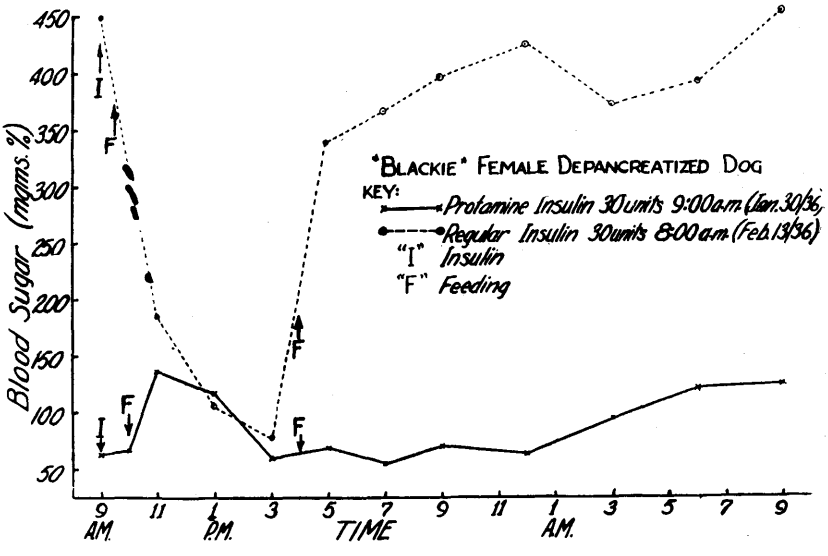
Dog	Regular Insulin		Protamine Insulin	
	Dose of Insulin	Sugar Excretion (Daily average 7 days)	Dose of Insulin	Sugar Excretion (Daily average 7 days)
"M"	17 units (q. a. m.)	40.9 g.	17 units (q. a. m.)	1.0 g.
"R"	26 units (q. a. m.)	13.2 g.	26 units (q. a. m.)	1.7 g.
"P"	34 units (q. a. m.)	56.1 g.	34 units (q. a. m.)	5.6 g.
"B"	30 units (q. a. m.)	48.3 g.	30 units (q. a. m.)	2.7 g.

dose of regular insulin (20 units) is given at one injection large amounts of sugar are excreted (Table 1, Kerr and Best). These results provide additional evidence that a large part of the insulin administered is excreted or otherwise lost when its absorption is very rapid. As in human patients the fluctuations of blood sugar throughout the day are largely eliminated and the incidence of hypoglycaemic reactions is very definitely less (Figs. 9 and 10). The results on diabetic dogs indicate that protamine insulin is a much more effective therapeutic agent than regular insulin.

HYPOGLYCAEMIA AFTER INJECTIONS OF PROTAMINE INSULIN

Observations on diabetic dogs have shown, in confirmation of clinical findings, that while the incidence of hypoglycaemic reactions is less with protamine insulin than with regular insulin those produced by the protamine preparations are often much more severe. No difficulties are encountered when the hypo-

glycaemia is treated immediately and plenty of sugar is made available. It is apparent that the hypoglycaemia produced by protamine insulin is more insidious in onset and, perhaps for this



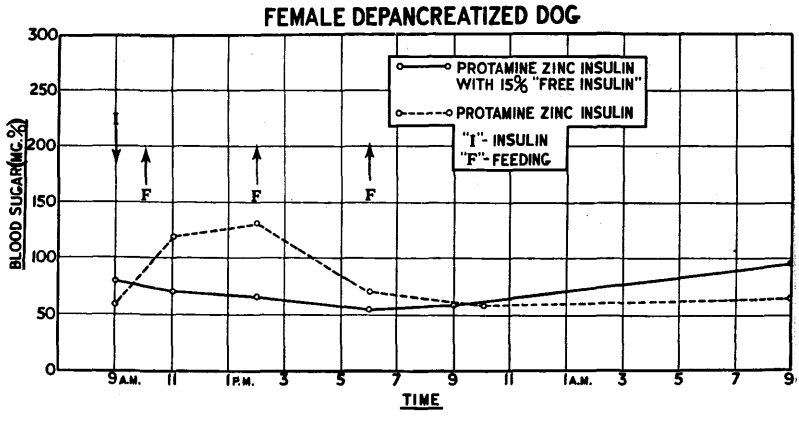


FIGURE 9 (upper); FIGURE 10 (lower), both from Kerr and Best.

reason, more difficult to correct than that produced by unmodified insulin. These findings emphasize the point that great care should be taken to prevent hypoglycaemia when protamine insulin or protamine zinc insulin is used.

DIET AND PROTAMINE INSULIN COMPOUND

Since the effect of one injection of protamine zinc insulin may persist for from 24 to 30 hours one dose per day may provide adequate insulin therapy in many clinical cases. This is the objective which most clinicians have in view. Since this preparation contains no appreciable amount of "free insulin" the rapid utilization of sugar very soon after administration, which is a characteristic effect of regular insulin, is not usually observed. Furthermore, the time after injection at which the lowest level of blood sugar is secured is much later than with unmodified insulin. For these reasons clinicians have found it necessary to make a redistribution of the food intake to provide larger amounts of carbohydrate later in the day. By this procedure a greater utilization of carbohydrate is secured and the incidence of the late hypoglycaemic reactions is decreased. The reader is referred to the clinical literature for details of the methods used.

SUMMARY

The results of Danish workers have made available a compound of insulin and protamine which exerts a prolonged anti-diabetic effect in cases of diabetes mellitus. Investigations initiated in the University of Toronto have shown that zinc may play an important rôle (1) in the stabilization of the protamine insulin compound and (2) in further prolonging the absorption of insulin. As a result of this series of studies a protamine zinc insulin preparation of satisfactory potency and stability, and contained in one vial, was made available for clinical trial. This product has been tested in many diabetic clinics and is now generally used. It is possible that this preparation may be further improved by the provision of a fixed amount of "free insulin" in the mixture. Some of the problems arising out of the use of these materials have been discussed.

Protamine insulin and protamine zinc insulin by enabling clinical and laboratory investigators to procure a more uniform and prolonged absorption of the anti-diabetic substance may assist materially in the investigation of many problems in carbohydrate metabolism.

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