I N S U L I N

Its assay, extraction and sources.

THESIS

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by

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C O N T E N T S.

PART I - INSULIN ASSAY.

The dose-response curve.

I. THE DOSAGE RESPONSE CURVE.

(a) Introduction and Discussion of Literature.

In May, 1922, Banting and Best, working in the Physiology Department of the University of Toronto, obtained the first active extracts from pancreas. The credit for the preparation of such extracts capable of effecting a lowering of the blood and urinary sugars, and serviceable in mitigating the symptoms of experimental diabetes in animals, and of human diabetes, therefore, belongs to these two workers, to Macleod and Collip (1). Improvements in the methods of extraction and purification of insulin followed quickly. Accompanying this were the investigations of the physiological, chemical and physical properties of the hormone. Since that time a mass of evidence relating to the above and produced by a host of workers has accumulated.

The most important and easily determinable physiological action of insulin is its effect in lowering the blood sugar when injected parenterally into animals. This is accompanied by other physiological disturbances and, when sufficiently great, it results in hypoglycaemic coma, convulsions and, finally, death, (4). These symptoms can be relieved almost instantaneously by the administration of glucose. Dogs, cats and rabbits show about the same degree of sensitivity; pigs are very sensitive (155) and sheep more resistant (156, 157). Goats are very resistant and require at least 4 units per kilo body weight to produce shock (158). Mice show little response at room temperature, except that the body temperature falls to a low level. If, however, the animals are

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kept at 28°C., they go into convulsions and coma (Krogh quoted by Macleod - 159). Fraser (8) showed that these animals are very sensitive to the action of insulin if injected intraperitoneally, provided they are starved prior to injection and kept at slightly increased temperature. According to McIntyre and Burke the albino rat normally shows a high resistance. Huxley and Fulton (161) found that convulsions develop much more slowly in frogs kept at low temperature (6-8°C - 120-140 hours) than in those at higher temperatures (30°C - 14 hours) and concluded that "the activity of insulin itself is not essentially altered by temperature, but that its speed of action is dependent upon the metabolic rate of the animal itself."

The effect of insulin preparations on the blood sugar in normal animals was first studied by the Toronto workers (4), who observed the fall in blood sugar level of rabbits with the injection of a sufficient dose and the occurrence of convulsions and coma, when the blood sugar had dropped to about 0.045 per cent. These findings formed the basis for their definition of the "Original Toronto Unit" which was given as "the smallest dose of insulin that would lower the blood sugar of a 2-kilo rabbit, starved for 24 hours, from the normal level of 0.118 per cent to the convulsant level of 0.045 per cent". However, this unit was modified by the Public Health Committee of the League of Nations to the "Clinical Unit", ie, "The unit of insulin is one-third of the amount of material required to lower the blood sugar of a 2-kilo rabbit, which has fasted 24 hours, from the normal level of 0.118 per cent to 0.045 per cent over a period of 5 hours."

The blood sugar fall was soon shown to be dependent on and affected by many factors: (i) The degree of

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response is dependent on the purity and source of the preparation tested (31, 162). (ii) Some rabbits are over-sensitive and convulsions appear before the blood sugar has reached the convulsive level of 0.045 per cent (162). (iii) The sensitivity of the animal to insulin action is variable (163, 161). (iv) The sensitivity of the animal depends upon its environment (7, 8, 159, 164).

With the injection of small doses, the rate of fall of the blood sugar is dependent on the method of injection. Subcutaneous injection results in a rapid decrease in blood sugar, which attains a minimum in 3 - 5 hours (165). The onset of the fall is later and its duration longer with intrathecal and subarachnoid injection than with intravenous (166). Subcutaneous and intravenous injection are equally effective (167). The actual value of the blood sugar depends on the sight from which it is drawn (168, 169). The capillary blood sugar corresponds to the arterial value, but the venous may be much lower (170).

The absolute value of the blood sugar varies with the analytical methods employed. The nature of the non-glucose reducing substances in the blood was investigated by Herbert, Bourne and Green (23, 171). The most important of these non-glucose reducing substances is gluthathione. According to Rabinowitch (24) the non-glucose reducing substances are responsible for 0.025 per cent of the reducing value of the blood and have a value 16-31 mg.per 100 ml. Ernst and Foster claim that the blood sugar at its lowest value in rabbits suffering from insulin hypoglycaemia has a reducing action, of which only 51-76 per cent is due to glucose (172). Hiller et al.(25) showed that, when inducing insulin shock in rabbits, though the reducing fermentable sugar was reduced to zero, the

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concentrations of non-fermentable reducing substances were not altered. Similar results were reported by Dotti (173). The value of the blood sugar and the significance attached to it, therefore, depend upon the method of estimation.

Administration of insulin to depancreatized animals restores the blood sugar to normal, the respiratory quotient rises, excessive ketogenesis and gluconeogenesis are inhibited and the glycogen stores are adjusted to normal. Pauls and Drury (175) administered insulin to fasted, depancreatized rats and found a marked augmentation in muscle glycogen (0.16-0.60 per cent) and also liver glycogen (3.26-9.78 per cent), which confirms the findings of earlier investigators.

Injection of insulin into intact animals results in less measurable and more complex physiological reponses. The free sugar content of the liver and kidney in starving animals is markedly lowered, but that of the muscles is not influenced (174). Insulin administration does not increase the utilization of carbohydrate in the organism as a whole (176). From the experiments of Bridge (177), it seems that the action of insulin is seen only in the distribution of glycogen between liver and muscle; without insulin most glucose is deposited as glycogen in the liver and with insulin relatively little goes to the liver, most appearing in the muscles. This confirms the work of earlier investigators (178, 179). Stetten and Klein (180) found that the glycogen appearing in the muscle after administration of insulin plus glucose is formed largely directly from glucose.

Whether the disappearance of glucose from the blood is the determining factor in the onset and severity of insulin convultions remains a question. Fischler and Hynd (quoted from 42) expressed the view that convulsions

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were caused through the formation of methylglyoxal and glucosone respectively in the tissues. Rudy (182) and Rabinowitch (183) postulated that the symptoms are due essentially to the disappearance of sugar from the blood stream. Another view is that convulsions are caused by the lack of oxidation in the central nervous system cells (184, 185). According to Gerrard (186) convulsions occur because there is no available carbohydrate for normal oxidation. Drabkin and Ravdin (187) postulate the following mechanism for the production of convulsions:- Hypoglycaemia → anhydraemia → rise in cerebral fluid pressure → unknown mediating factors, possibly nervous → convulsions.

Since the successful preparation of insulin and its subsequent manufacture by commercial firms for therapeutic use, the necessity has arisen for an accurate and reliable method of assay. Although a physical method (2), based on the absorption bands of insulin as criterion of its activity and a chemical one (3), based on the inhibition of oxidation of polyhydroxyphenols by hydrogen peroxide in the presence of insulin, have been proposed, these have both been followed up without success. The result is that, up to the present time only the biological method of testing insulin activity has proved applicable.

The early workers tested the activity of their preparations by injection into animals and noting the degree of effect, by which procedures the conception of "Original Toronto Unit" and "Clinical Unit" originated.

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Thus a direct method of determining insulin potency, based on the fall of the blood sugar in rabbits after the subcutaneous injection of varying quantities of an insulin preparation, or upon the incidence of convulsions in rabbits (5), had come to be used by different laboratories. This direct method was extended to other labotatory animals, white rats and mice being used for determining insulin activity by the percentage drop in blood sugar (6) or by the incidemce of convulsions (7, 8).

However, it was soon found that, mainly because of the wide variation in response in experimental animals, the direct method was inaccurate. Meanwhile a standard was adopted in 1925 by the Standardization Committee of the League of Nations (9), this being a special preparation of dry insulin hydrochloride regarded as containing 8 units per milligram. In 1935 this old international standard was replaced by the new international standard by the Committee (10), this being a recrystallized preparation of crystalline insulin to which has been assigned a potency of 22 international units per milligram.

As shown previously, the factors which affect the response to insulin administration, are many and varied. These obviously restrict the accuracy of the biological assay of insulin. In order to minimize or exclude as many of these, the indirect or comparative method of testing was introduced. In this the effect of the unknown sample is compared with the effect of a known strength of insulin standard and expressed in terms thereof. To compare the activity of insulin preparations with the standard, two procedures have come to be generally and almost universally employed, viz:- (1) the method based on convulsions in mice and ((ii) the method

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based on the fall of the blood sugar in rabbits, both of these having been fully described and discussed by various authors (11-17).

The use of mice for this purpose was first suggested by Fraser (8) and Voegtlin and his collaborators (164). Trevan and Boock (17) forwarded the most practical method, while various refinements have been added since it was first devised (15, 16, 118, 189).

The starting point for the development of this method was the observation that mice, kept at room temperature, were able to withstand large doses of insulin, but were promptly killed by small doses when kept in an incubator at temperatures ranging from 25° - 35°C (8, 159). The method, as described by Hemmingsen and Krogh (15), was first used as a direct method and the mouse unit was defined as that amount of insulin that would produce convulsions in half the number of mice injected. The relation between the mouse unit and the Original Toronto Unit (4) was found to be of the order of 600:1.

From 3 to 10 mice, weighing from 16 to 20 gm., were used for each assay. They were fed on bread and water and each animal injected subcutaneously with the suitably diluted insulin to be tested. They were then placed singly in glass jars in an incubator with glazed doors and the temperature maintained at 30°C. According to the incidence of convulsions, the activity of the sample under test was calculated in terms of mouse units. Due largely to fluctuations in sensitivity, the numbers of test animals were greatly increased and the comparative method reverted to (loc. cit.), where the activity of the sample was compared with the activity of the standard (private standard of the Nordisk Insulin Laboratorium, one unit being 0.085 mg. as compared to 0.125 mg. of the

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international standard). From 60 to 80 mice were used, half receiving the standard and half the unknown sample and each test repeated from 8 to 12 times with different concentrations on 4 to 6 consecutive days. The number of mice convulsing in each group for each test were noted, and the final result obtained by plotting, for standard and unknown, the percentage convulsions against doses. From the two curves thus obtained, the activity of the unknown, in terms of the standard, was calculated arithmetically. Subsequently the curves were replaced by straight lines by plotting the percentage convulsions in each test against the logarithmic values of the doses, and the final result obtained graphically or arithmetically by the method of least squares. It is important that in each test the assumption regarding the strength of the unknown should be as nearly correct as possible, so that about the same percentage convulsions is obtained in each group. Each test serves as a guide for the next and, by finding the probable strength of the unknown by interpolation in the normal curve and using this result in the next test, reliable results can be obtained, even with large simultaneous variations in the general sensitivity.

Trevan and Boock (17) fed their mice on white bread and excess milk and starved them for 12 to 18 hours prior to the test. Groups of 60 animals are used, of which half receive the standard and half the unknown, doses being adjusted to a body weight of 20 gm. During the test the animals are placed in zinc boxes, which are for three quarters of their height immersed in a water bath at 38°C. In all, four tests are made using the same dosage and the number of animals convulsing within two hours with each test observed.

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The percentage convulsions for each group is platted against the doses and the two curves compared with a predetermined reference curve, from which can be calculated the ratio of the activities of the standard and sample under test. Trevan considers that the relation between percentage increment of dose and percentage increment of convulsion rate is constant, which means constancy of the actual slope of the log. dose - convulsion rate curve, except for variation due to sampling of the mice.

Following the contention of Trevan that a temperature of 29°C is less effective for performing the test than higher temperatures because the dose-convulsion rate curve is too flat (17), Hemmingsen and Marks (188) compared the effectiveness of different temperatures and found that the distribution of convulsive doses for mice at 37°C is similar to that at 29°C and that the slope of the average line relating to the normal equivalent deviation of the percentage convulsion rates to the log.dose at 37°C, does not differ much from that at 29°C.

Hemmingsen (16, 190) studied the distribution of individual sensitivities of mice, the factors influencing sensitivity and the variation in the slope of the log.dose-convulsion rate curve in a large number of animals, subjecting the results to statistical treatment. He found that the individual sensitivities of mice to insulin are normally distributed (logarithms) and that the true slope of the log.dose-normal equivalent deviation cannot be regarded as constant. He described a method (16 p. 192) in which the slope of the log.dose-normal equivalent deviation relation is indirectly assessed by means of the differences in assumptions of different tests, and in which one dose of each preparation is so injected that the influence of possible changes in sensitivity during the single tests is eliminated as far as possible.

A disadvantage of the mouse method is the subjective

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selection of animals showing symptoms of hypoglycaemia. To overcome this and, at the same time, reducing the cost of the assay and personnel requirements, Thompson (194) introduced the sloping screen technique. The groups of injected mice are placed on 60° sloped wire mesh screens. They remain on the screen until advanced insulin symptoms cause them to lose their foothold, falling free from the edge of the screen into the provided trays, from which they can be removed for injection with glucose. However, it was found that the mice do not fall away from the screen until signs of insulin shock are far advanced, so that the mortality is high. Young and Lewis (195) described an improved unit for this purpose, consisting of hollow wire mesh cylinders mounted at an angle of 60° on wooden rollers, whereby the rotation assures that the foothold is never secure for long and, at the first signs of insulin shock, the mice fall into trays beneath the cylinders. These contain chow pellets and the animals are able to eat sufficient food to relieve their hypoglycaemia, so that injection with glucose is unnecessary. With this procedure the mortality was reduced from 7-10 per cent to 1.7 per cent.

The principles involving the bio-assay of insulin by the rabbit method, as it was originally employed, have been discussed by Macleod and Orr (191) and the report by the Insulin Committee of the University of Toronto submitted (loc.cit.). The earlier workers (4) had determined insulin activity in terms of a convulsive level at a blood sugar concentration of 0.045 per cent (c.f. original Toronto Unit). It, therefore, became necessary to choose between the incidence of convulsions and the fall in blood sugar level as basis for the method of assay For reasons supplied by the writers (loc. cit.) the Insulin Committee of the University of Toronto adopted

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the fall of the blood sugar. Their method of performing the assay was as follows:-

To get an approximation of the potency of the unknown sample, ± 18 rabbits are injected with a widely varying number of doses (± 6) and the lowest dose, producing convulsions in the majority of animals, observed. This convulsant dose of the unknown sample is then considered = 1 original Toronto Unit = 3 clinical units, and on this value is based the amounts of the unknown sample to be injected into the test animal. For each test 9 rabbits, weighing from 1.8 - 2.2 kg. are used. They are starved for 24 hours and, immediately before injection, sufficient blood is collected from each animal to determine its initial or fasting blood sugar level. Three rabbits are then injected with an amount of the unknown sample estimated to contain 2. 5 clinical units, 3 animals receive an amount estimated to contain 2 clinical units and the last three 1.5 units. Injections are made subcutaneously and from each animal blood is drawn at 1½, 3 and 5 hours after injection. The blood filtrates for a particular rabbit are mixed or pooled, so that only one sugar titration is necessary for each rabbit. The activity of the sample is then calculated from the equation:

$$\text{Activity (clinical units per cc)} = \frac{a}{b} \times \frac{w}{c} \times 1.5$$

where a = percentage of blood sugar before injection minus the average of the percentages of blood sugar found in the samples taken 1½, 3 and 5 hours following the injection;

b = percentage of blood sugar before injection minus 0.045 per cent;

w = weight of rabbit in kg;

c = number of cc. of the original (undiluted) insulin solution injected.

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Using this direct method, the old international standard (insulin hydrochloride) assayed at 8.1 units per mg.

Marks (14) described the comparative method, as it was evolved and used in the National Institute for Medical Research, London. As reference standard was used, a sample of insulin solution supplied by Messrs Eli Lilly & Co.and standardized by the Toronto Insulin Committee. At least 6 rabbits are used for each assay. They are starved for 24 hours and blood for the determination of the fasting level withdrawn immediately before injection. Half the animals of the group are then injected with 1 clinical unit of the reference standard per 2 kg.bodyweight and the other half are given a theoretically corresponding dose of the unknown sample. From each rabbit blood is taken at hourly intervals for five hours and the samples from an individual animal pooled. Titration of this sample gives the mean value (average = absolute level) of the blood sugar over 5 hours and subtraction of this from the fasting or initial value, gives the absolute blood sugar reduction, which is then converted to the percentage blood sugar reduction in order to compensate differences in initial blood sugar values.

To further eliminate errors introduced by individual differences and periodic fluctuations in the response of the rabbits, the test is repeated a few days later, but the groups are reversed - the so-called crossover - so that those rabbits, which previously received the standard, now receive the unknown and those that had received the unknown receive the standard. In the same way as previously, the percentage blood sugar reduction for each animal is determined. The sum is now found of all the figures for the two days relating to the unknown sample, and similarly also the sum of all the figures relating to the standard. A comparison of the two totals then dndicates the approximate activity of the unknown in terms of the standard,

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which can then be calculated. In a series of ten tests the standard error was found to be 7.0 for the crossover method as compared to 9.9 for the direct method (loc. cit.).

Applying this method to the evaluation of the old international standard, a value of 9.3 units per milligram was found. The application of statistics to this method has greatly increased its accuracy.

In 1939 Bliss and Marks (33) introduced the latin square cross-over design, and applied the analysis of variance and co-variance to the assay of insulin as a means of studying the variables affecting the precision of the method of assay. However, this method suffers from the drawbacks that, for routine assay, it takes longer to complete than the ordinary crossover, and that the occurrence of convulsions or deaths complicates the arithmetic. In order to overcome some of these difficulties, Fieller (34) in 1940 proposed the split cross-over design, which consists in effect of two cross-over tests carried out simultaneously, the doses of the standard being the same in both, but those of the test different. This method supplies an estimate of the log dose-response line, but it has the disadvantage that the estimate of the slope is obtained by varying the doses of only one of the two preparations, standard and test. In 1944 Smithetal (35) extended the work of Fieller to the twin cross-over design in order to further simplify the procedure and rule out this disadvantage. This, like the split crossover, consists of two simple crossovers, in the one a high dose of the standard being compared with a low dose of the test, and in the other a low dose of the standard with a high dose of the test.

Lacey, in 1941 (192), described the method of insulin assay used in the Insulin Committee Laboratory, University of Toronto, and subsequently adopted, with minor modifications, as the official method by the United States Pharmacopoeial Convention. This 3-assumption crossover

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method, involves testing the material on the bases of three assumed values (as opposed to the single assumption of the potency of the unknown inother tests), all these values being used on each day of the test. Three separate and complete cross-over tests are, therefore, made, using from 30 to 40 rabbits for each test. The range of assumption should be as narrow as possible. In a case of material purported to contain, say, 20 units per cc, dilutions for injection are made on the bases of 18, 20 and 22 units per cc.and each of these dilutions compared with the standard in an ordinary crossover. The three assay results are graphed, plotting assumed potency against result and the true potency read off directly. The test lasts over a period of 5 hours with a bleeding schedlue of 0, 1.5, 3 and 5 hours. The doses administered should be sufficiently large to ensure an average blood sugar level below normal throughout the test period, but not so large as to cause maximum effect.

In a subsequent paper (1946) the same author (193) pointed out that, for a satisfactory assay by means of this method, the range of assumed values should be very small. Its useful range is very limited and, by means of the twin crossover design, it is possible to evaluate potency over a much wider range. It can, therefore, be successfully applied only when the material under test has been carefully "bracketed", ie., its potency determined by some other means to within 10-20 per cent of the actual value. The author (loc. cit.) subsequently presents modifications to be applied to both the 3-assumption crossover and the twin crossover methods. These consist of following a 0, 2, 3 and 4-hours bleeding schedule and the injection of the insulin in clinical dosage forms, as it has been shown that the blood sugar curves are different, following

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administration of various dilutions of insulin to rabbits, even though the unit dose per rabbit remains unchanged. With increasing concentration there is a progressive increase in rate of action and decrease in duration of effect. Injection in clinical dosage forms involves amounts as small as 0.01 cc. and requires the use of a micrometer syringe.

Thus far the doses of insulin had been administered subcutaneously in all assays. Young (1945) studied the dosage-response relationship after intravenous administration of the doses and, with Romans (29) reported a method of assay depending on intravenous injection of the doses and a single bleeding 50 minutes after injection. From the results of 102, twelve rabbit assays they could detect no significant variation in the slope of the dosage response curve over a period of 14 months, and found the standard error to be of the order of 11 or 12 per cent. Pugsley and Rampton (30) compared the potency of commercial insulin preparations, when assayed by the method of subcutaneous administration and the method of intravenous injection (Young and Romans), and found that the latter method gives results in good agreement with those obtained by the former, as regards the slope of the regression line and the standard error.

The generally-recognised methods of insulin assay have been compared by Young et al.(36), who found a standard error of the estimate of potency of 13 per cent for a 32-rabbit subcutaneous assay, 10 per cent for a 16-rabbit intravenous assay and 9 per cent for two 144-animal mouse assays.

The use of other laboratory animals for insulin assay was introduced by Opdyke (196), who reported that the chick offers possibilities as a test animal for insulin assay. He found that the blood sugar of the

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chick is lowered in response to light doses of insulin, and the duration of the hypoglycaemia shortened by increasing the dose. The percentage decrease of blood glucose 1½ hours after injection is a straight line function of the logarithm of the dose up to about 1.5 units per kg. Although the individual variation in response is not excessive, the author subsequently (197) tried to reduce this by prolonged fasting and dehydration. White Leghorn cockerels between 30 and 40 days old and body weight varying between 200 - 400 gm., were used. After fasting periods of 14, 24, 48 and 72 hours some were injected intramuscularly with 0.1875 unit of insulin, the others acting as controls. In the control groups it was found that the blood sugar rose after the 14thhour, and continued to rise through 72 hours of fasting. This rise was augmented by dehydration so that the blood sugar rose more promptly and to higher levels. It was found that neither fasting alone nor fasting plus dehydration affected the insulin sensitivity, and that fasting plus dehydration resulted in a marked decrease in the variability of the blood sugar values, whereas fasting alone increased variability. The variance in blood sugar values 1½ hours after injection was least in chicks fasted and dehydrated for 48 hours. The author concludes that the precision of the results, after 14 hours of fast, is sufficient for routine insulin assay by the chick method.

The detection and measurement of the insulin content of the body fluids has long been a difficult and almost impossible task. Hemmingsen et al.(198) had shown that adrenalectomised mice develop hypoglycaemic convulsions on doses of 0.0001 to 0.0002 unit of insulin per 10 gm. of body weight. However, their criterion, viz: convulsions, did not permit them to

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assay insulin very accurately, because of the marked individual differences in the convulsive threshold. Feldman et al.(199) suggested the hypoglycaemic effect as index, and found that minute quantities of insulin could be detected in the blood of adrenalectomised rats which had been subjected to anoxia or metrazol. Gellhorn et al. (200) subsequently used hypophysectomised, adreno-demedullated and hypophysectomised-adreno-demedullated rats for the assay of insulin by itself and in the presence of blood. They found that 0.025-0.03 unit insulin per 100 gm.of body weight causes convulsions in the hypophysectomised rat, 0.005 unit in the adreno-demedullated animal and 0.001 unit in the hypophysectomised-adreno-demedullated rat. Extending this work (1947), Anderson et al. (201), using adreno-demedullated diabetic hypophysectomised rats, found that 0.00012 unit of insulin, administered intravenously, causes a sufficient lowering of the sugar for this method to be used for detection of insulin in blood.

Notwithstanding the utmost care as regards selection of animals, feeding, housing, etc., it has, as yet, not been possible to exclude the wide variations in fasting blood sugar values which are frequently encountered. By statistical analysis of a restritted number of data de Jongh (26) found that the initial blood sugar level has, within wide limits, almost no influence on the absolute value of the decreased level caused by insulin injection. However, de Leeuw (27) observed larger differences and he calculated a correcting factor, by means of which they could be eliminated. Marks and Hemmingsen (13) have shown that both the absolute and percentage fall increase with increasing initial value, and, through statistical analysis of a large amount of material, came

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to the conclusion that the percentage fall increases by approximately 0.22 for an increase of 1 mg. per cent in the initial blood sugar value. On this observation they likewise based their method of correcting the results of tests for differences in initial blood sugar level. In a later communication (1934) Marks presented a slightly higher regression coefficient, viz:0.025. In 1947 de Jongh, Lens and Spanhoff (28), working on a large amount of accumulated material, obtained a regression coefficient of 0.032 (for every 0.001 °/oo increase in initial blood sugar level, the relative decrease increases with 0.032%). However, the value of the regression coefficient found in a series of experiments undertaken at different times has been shown to vary (28).

In contrast to the importance attached to variations in the initial blood sugar level and the subsequent determination of the regression coefficient in order to obtain the correction factor, are the findings of Young, 1945 and Young and Romans, 1947 (29). These authors performed 102 twelve-rabbit insulin assays according to the intravenous method. The initial sugar determination was omitted and no significant variation in the slope of the dosage-response curve was detected over a 14-month period. Pugsley and Rampton (30) have similarly found that, in both the subcutaneous and intravenous methods, the initial sugar value may be excluded from the calculations without affecting the precision of the assay.

The effect of insulin has been expressed differently by different workers. Marks (14) assumed that the absolute average fall of the blood sugar, after injection of a certain dose of insulin, is proportional to the absolute value of the initial blood sugar, and, therefore, expressed the blood sugar fall as a percentage of the

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initial blood sugar - the percentage reduction. Krogh and Hemmingsen, 1928, adopted the absolute fall as the measure of effect, whereas Laqueur (31) ignored the initial blood sugar and merely observed the lowest point to which the blood sugar fell during the test. In a later communication (13) Hemmingsen and Marks submitted a large amount of material to statistical treatment and found that both the absolute reduction and percentage reduction increase with increasing initial value. de Jongh et al. (28), through similar treatment of accumulated material, obtained corresponding results, and conclude that calculations can be based with equally satisfactory results on either the absolute reduction or the percentage reduction (after application of the correction factor for initial values). Pugsley and Rampton (30) have similarly found no significant differences when basing their calculations on either the percentage reduction or absolute reduction. In the intravenous method (Young and Romans, (29)) the initial value is disregarded and the calculations based on the absolute decreased level.

It has become customary to measure the effect of insulin on the blood sugar by means of the average fall (absolute or percentage) during the first four or five hours following the injection. This period was originally decided upon, as a result of investigations carried out by the earlier workers (14, 191), as of sufficient duration for the blood sugar to return to its normal value, following the injection of the specific insulin dose employed for assays. The bleeding schedule followed by most workers is that of 0 (initial), 1.5, 3 and 5 hours; others (28) prefer a 0, 0.75, 1.5, 2.25 and 3 hours schedule. As regards the bleeding schedule, Bliss and Bartels, using the discriminate function test, claim that "the different time intervals are markedly unequal

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the information they provide on the insulin effect, and the application of this method to more extensive data should provide a revised bleeding schedule, which would be both simpler and more efficient". Pugsley and Rampton (30) have compared the effect of variations of the bleeding schedule on the slope of the regression line and the standard error. Their results indicate that, in the conventional bleeding schedule of the subcutaneous method, the 5-hour sugar value does not contribute to the precision of the assay and may, therefore, together with the 0 sugar value, be excluded. Similarly, sugar determinations can be made at 2 and 4 hours only or at 1.5 and 3 hours only, without seriously affecting the precision of the assay. In the intravenous method Young and Romans (29) make a single sugar determination at 50 mins. after injection.

A surprising factor in the assay is the meagre contributions by research workers on the amount of insulin dosage and possible variations of the dose. This is probably due to the long practised, conventional bleeding schedule of 0, 1.5, 3 and 5 hours where a dose of $\pm$ 1 I.U. per 2 kg. body weight has been found to give the desired result. The Toronto workers (191) used a subconvulsive dose of 2.6 clinical units per 2 kg. body weight. Marks (14) investigated the relation between dosage and effect and found that, on the lower doses, the hypoglycaemic effect is approximately proportional to the dose, but as the doses are increased, the hypoglycaemic effect reaches a limit, above which there is practically no further increase, however large the dose. It is, therefore, essential to employ a dose smaller than 1.50 units per 2 kg. in order to obtain hypoglycaemic effects varying approximately with the dose. "On the other hand, the dose should be as large as possible, so that the effect shall not be so small as to give undue prominence to

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experimental errors. For this purpose a dose of 1 unit per 2 kg. has been adopted" (loc. cit.). Nevertheless, it is possible that experimental variation of the dose, especially towards the lower ranges, may yield equally fruitful results as have been obtained by variation of the bleeding schedule. With the introduction of the split cross-over and the twin crossover designs, the dose has come to be varied, ranging from 0.3 units per kg. for the low dose to 1.6 units for the high dose, according to the sensitivity of the individual animals (35). Other workers have used higher dosage levels, being as much as 0.90 units for the low and 1.80 units for the high dose per rabbit (30). The continental workers (28), using a 0, 0.75, 1.5, 2.25 and 3 hours bleeding schedule, have been working on slightly lower dosage levels. The effective dosage levels in the intravenous method are within the same range as those required for the subcutaneous method.

Previous studies (14) have shown that over a fairly wide range of doses the effects of insulin injection are proportional to the doses, plotting of these results, i.e., dosage against average percentage blood sugar reduction, yielding the dose-response curve. Later studies indicated that for part, at least, of the effective range this relation is linear, when the percentage reduction is plotted against the logarithm of the dose. Bliss and Marks (33) have considered at length those factors by which the precision of the curve, relating dosage and graded response, can be increased. They indicate that "when the relation between dosage and response is to be used subsequently for purposes of biological assay, it is advantageous to find the measure of response which will plot as a straight line against the logarithm of the dose. If tested over a wide enough range of doses, most

/responses

responses show both minimal and maximal limits that are approached asymptotically, but an intermediate range is often present which is indistinguishable experimentally from a straight line and quite adequate for pharmacological assays." As regards the estimation of relative potencies by the slope of a predetermined curve, the same authors in a subsequent paper (37) point out that the slope of the curve may vary from laboratory to laboratory or from one test to another. This point was further elaborated on by Fieller et al.(38), who considered whether the dosage reponce relation should be determined separately in each assay, or whether it should be predetermined in a special experiment, and the result accepted as a permanent standard of reference for use in all subsequent assays. They compared curves obtained from two series of assays from different laboratories and spaced by an interval of 10 years and the curve obtained by Bliss and Marks (33), finding a satisfactory agreement between the slopes in the three curves, the slopes b' being = 53.6, 46.7 and 46 with standard errors of $\pm$4.9 $\pm$ 4.8 and $\pm$ 2.7 respectively. They came to the conclusion that a log. dose-response relation with a slope of 45 is appropriate. In a subsequent paper (39) the same authors compared the dosage-response lines for 3 laboratories, two of these being commercial ones, where the work consisted of routine assays on a large number of different preparations and found, especially in the case of the latter laboratories, that real changes in the slope occur relatively frequently. They conclude that "a dosage-response relation obtained by means of a special experiment in a particular laboratory may not necessarily apply strictly to general routine assay and it would, therefore, be desirable for any laboratory in which assays are regularly carried out to analyse its own experience. A further improvement would be to arrange each separate test so that it should provide a fairly

/reliable

reliable estimate of its own slope." Both the split crossover and, especially the twin crossover, due to the differences in dosage, fulfil this requirement, so that, in an assay of the latter design, the use of a predetermined log. dose-response line has become unnecessary and superfluous.

The majority of the reports on the assay of insulin aim at increasing the precision of the method, being concerned either with the effect of variations in the design, or with methods of interpreting the results. Precision of the assay is, therefore, of fundamental importance when dealing with purified material to be used for pharmaceutical or research purposes. However, the research worker in the field of insulin extraction by various methods, or from different sources, is confronted by the problem that he requires a quick estimate of the potency of an insulin containing sample of varying degrees of purity at different stages of the extracting procedure. For obvious reasons the methods of assay as explained in the previous paragraphs, do not fulfil this requirement.

First and foremost, these methods are too precise for the purpose in view involving an unnecessary complication of procedure and calculations. Often the material that is tested is so impure, as compared with the standard, that a full assay would yield no more enlightening results than could be obtained through simpler procedure. This was the case with many of the samples quoted in the subsequent sections of this work. The worker in this field is interested to know whether a certain source yields, say, 1,000 or 3,000 units per kg. of material, or whether

/a

a certain procedure increases the yield of his product from, say, 500 units to 1,000 units per given weight. He is not concerned, at this stage, with the exact activity of his ultimate purified product, but needs a quick method and yet one whihh is sufficiently reliable to give him an approximate estimate of the potency of the material in hand. The standard methods of assay can be compared to the fine adjustment of a microscope, the specifi aim and requirement being clarity and precision. However, it can be assumed that each laboratory, where insulin assay is undertaken as routine for or specifically, also has a "coarse adjustment" by means of which the activity of an altogether unknown sample is first roughly determined before switching over to the fine. Descriptions of these "coarse adjustments" shine by their absence from the literature.

A second disadvantage is the duration of the properly-conducted rabbit assay. Depending on the numbers of animals used and the specific design followed, this assay may require from 1 to 4 weeks to complete. If 8 or 12 animals are used and the twin crossover employed, at least a week is necessary for completion of the test, the conventional bleeding schedule not permitting feeding of the animals in between, and thus preventing the test running over four consecutive days. This method serves the purpose very well when performed for research purposes aiming at increasing its precision or practicability, or when single large batches of material are tested, as is done in commercial laboratories, but it is impractical when large numbers of samples require testing. The intravenous method, by allowing feeding of the animals in between, shortens this period to 4 days and, therefore, has distinct advantages over the subcutaneous method.

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In the mouse method large numbers of experimental animals (varying from 150 - 200 per assay) are required. This necessitates the carrying of large stocks over long periods; In many institutions, where the research fields of their various members are of a diverse nature, the costs attached to the maintenance of adequate numbers of animals, cause the mouse method to be an expensive and impractical one.

In the latter sections of this work (see Parts II and III) large numbers of insulin containing samples (running well into three figures) had to be tested. For the reasons already quoted, the standard methods of assay would not only have been superfluous but would have made the task impossible. It was, therefore, decided to embark on a search for a "coarse adjustment", the features of which would be ease and rapidity of working combined with the maximum amount of precision that can be expected of such a method. In the following pages is described the procedure followed in order to arrive at a satisfactory solution. Apart from this primary aim, a secondary consideration was the possible instructive conclusions that could accrue from experimental procedure of this kind. The method, as it was finally adopted, was used in a quick assay of all the samples, but it by no means replaced the accurate full assay methods, which were reverted to in all cases where final exact determinations were required.

(b) Material and Methods.

The rabbits used in this test were of a mixed breed, having been bred from the local stock. On special instructions the young animals were frequently handled by the attendant, so that by the time they were used for the test, they had become accustomed to handling and were

/quite

quite tame. Before the animals in the group were selected, the entire batch was on two occasions injected with 0.5 unit insulin standard per kilo body weight, and the blood sugar determined at hourly intervals over a period of three hours. In each instance the average percentage reduction for each animal and the group was determined, and those that had shown insensitiveness or over-sensitiveness on both occasions were consequently omitted.

The experimental animals were housed in large cages - 3 to 4 animals to a cage of approximately 16 square feet floor space, and males and females kept separately. No special provision was made for temperature regulation in the animal house, the construction of this being such as to maintain as far as possible a fairly constant temperature. Moreover, the greater part of the test was performed during the warmer season of the year (September - April) and the laboratory temperature was hardly ever observed to fluctuate beyond the limits of 20 - 24°C. Occasionally, when lower or higher temperatures were registered, no evidence could be found of any deviation as far as the fasting blood sugar level was concerned, or the effect of the injected insulin, this being in agreement with the earlier observations of Scott and Dotti (18).

Throughout the duration of the test the rabbits were fed on a diet of cabbage, carrots, dried lucerne and oats. Prior to the injection of insulin they were placed singly into smaller cages and starved for periods varying from 14 to 18 hours, water being always available.

The weights of the animals varied from 1.6 to 2.8 kilograms and throughout the dose was adjusted to a mean body weight of 2 kilo. As almost all the animals received the entire dosage range, adjustment of dose to body weight seems to have been an unnecessary procedure. However, in

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the light of the ultimate use the results were to be put to, it seemed more expedient to follow this course.

Altogether 70 rabbits were used, a group of 42 being selected for each dose. It is, therefore, apparent that most animals were used for the entire range of dosage. Where accident to a rabbit or pregnancy of a female prevented it from being used, its place was taken by a new one. With the higher doses rabbits occasionally convulsed; these were then replaced by non-convulsing ones.

An average of 9 rabbits were tested per day, so that, with few exceptions, no animal was used more than once weekly, the test period having lasted approximately 18 weeks.

The contents of an ampoule of insulin standard was dissolved in distilled water containing 0.85 per cent sodium chloride, hydrochloric acid to make pH 2.5 and 0.3 per cent tricresol. The volume of solvent was such as to give 10 units per ml. This stock solution was stored in a refrigerator at 4°C. Immediately before injection the stock solution was diluted with a further volume of solvent to the required strength. The volume injected was 0.5 ml. per 2 kilo. bodyweight. The doses varied by 0.05 units and ranged from 0.05 - 0.85 units per 2 kilo. Altogether then, 17 groups of 42 rabbits each were tested. The different groups were treated at random and not according to a systematic schedule.
To illustrate this:
On the first day of the test 3 of the 9 rabbits received 0.45 units each, 3 received
0.70 units each, and 3 received
0.55 units each.
On the second day 3 received 0.15 units each,
3 received 0.35 units each,
3 received 0.50 units each,

/and

and so on until all 17 groups had been completed.

Injections were made under the loose skin of the flank. Immediately before the injection each rabbit was bled in order to determine the fasting sugar value, blood being obtained from the outer marginal vein of the ear. About 1 ml. of blood was collected from each rabbit into a small glass tube containing a very minute quantity of potassium oxalate. After injection the animal was put back into the cage and the latter placed in a dark cupboard, so as to exclude the disturbing factors of normal laboratory routine. The rabbits were removed from the cupboards only for short periods in order to draw blood. During the injection, but especially during the bleeding process, the utmost care was exercised to prevent undue activity of the animals such as jumping or jolting. The small size of the cages (12" x 18") prevented marked muscular activity of the animals while in the cupboards. Thorp (40) in 1944 described the effect of mild exercise upon rabbits which had received insulin, and showed that, in exercised animals, the blood sugar not only did not reach the same low level as in non-exercised ones, but it also returned more rapidly to normal. He also found the degree of animal variation to be higher in the exercised rabbits. When occasionally such muscular activity did occur during the bleeding, the results from the particular rabbit were discarded and a new one substituted in its place.

Blood was collected from each rabbit at hourly intervals over a period of three hours following the injection of insulin. The blood samples were not pooled, but each determined separately, so that the hourly level for each rabbit could be observed. Each sugar level listed in the succeeding tables represents the average reading of three separate determinations.

/The

The procedure used for the estimation of sugar was Harding's modification of the Schaffer-Hartmann method (19-21).

(c) Results.

In the following Tables 1 - 17 are listed the effects of varying doses of insulin standard on the blood sugar level of fasting rabbits over a period of 3 hours.

/TABLE I.

TABLE 1.

Dose: 0.05 I.U/2 kg. rabbit.

Serial Number of Rabbit	Fasting Blood Sugar level	Abs.fall at end of 1st hour	Abs.fall at end of 2nd hour	Abs.fall at end of 3rd hour	Average absolute fall over 5 hours.
1	69	8	-	-	3
2	74	6	-	-	2
4	58	-	-	-	-
6	66	14	-	-	5
7	68	16	-	-	5
8	78	9	-	-	3
9	57	-	-	-	-
10	80	-	-	-	-
11	63	4	-	-	1
12	84	-	-	-	-
15	56	-	-	-	-
16	67	6	-	-	2
17	73	7	-	-	2
18	76	-	-	-	-
20	54	5	-	-	2
21	63	6	-	-	2
25	82	12	-	-	4
26	79	9	-	-	3
27	70	-	-	-	-
28	65	8	-	-	2
29	64	13	-	-	4
30	76	-	-	-	-
32	59	10	-	-	3
33	62	4	-	-	1
34	65	4	-	-	1
35	66	-	-	-	-
36	77	15	-	-	5
38	72	3	-	-	1
39	70	-	-	-	-
41	64	11	-	-	4
42	58	-	-	-	-
43	81	-	-	-	-
44	58	-	-	-	-
45	68	8	-	-	3
46	78	17	-	-	6
47	82	13	-	-	4
48	80	-	-	-	-
49	70	5	-	-	2
50	57	-	-	-	-
51	72	12	-	-	4
52	74	3	-	-	1
53	79	5	-	-	2
TOTAL:	2914	233	-	-	77
Average:	69.4	5.5	-	-	1.8

TABLE 2.

Dose: 0.10 I.U/2 kg.rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs.fall at end of 1st hour	Abs.fall at end of 2nd hour	Abs.fall at end of 3rd hour	Average abs.fall over 3 hours.
1	68	6	-	-	2
2	82	27	2	-	10
3	73	-	-	-	-
4	68	-	-	-	-
5	72	14	-	-	5
6	74	22	-	-	7
7	82	28	5	-	11
8	84	25	4	-	10
9	71	-	-	-	-
11	66	-	-	-	-
12	84	25	9	-	11
13	58	-	-	-	-
14	61	8	-	--	3
15	60	2	-	-	1
16	85	18	10	-	9
17	82	18	-	-	6
18	67	16	2	-	6
19	77	26	-	-	9
23	66	8	-	-	3
24	85	30	10	-	13
25	74	3	-	-	1
26	74	8	-	-	3
27	76	21	2	-	8
28	63	-	-	-	-
29	78	-	-	-	-
32	72	16	-	-	5
33	78	20	2	-	7
34	76	23	-	-	8
35	66	-	-	-	-
36	72	-	-	-	-
37	86	18	6	-	8
40	54	-	-	-	-
41	65	11	-	-	4
42	68	3	-	-	1
43	83	17	-	-	6
44	80	16	-	-	5
46	85	28	11	4	14
47	70	3	-	-	1
49	60	4	-	-	1
50	66	12	-	-	4
52	56	5	-	-	2
53	64	-	-	-	-
TOTAL:	3022	481	63	-	182
Average:	72.0	11.5	1.5	-	4.4

TABLE 3.

Dose: 0.15 I.U/2 kg.rabbit

Serial Number of Rabbit	Fasting blood sugar level	abs.fall at end of 1st hour	Abs.fall at end of 2nd hr.	Abs.fall at end of 3rd hr.	Average abs.fall over 3 hours.
3	78	7	6	-	4
4	65	27	12	-	13
5	71	11	7	6	8
7	60	29	20	-	16
8	66	21	18	-	13
9	66	17	2	-	6
10	84	31	7	-	13
11	74	25	11	6	14
12	56	26	1	-	9
13	69	24	-	-	8
14	68	36	30	10	25
15	74	18	7	-	8
16	69	40	26	12	28
17	82	31	13	8	17
19	73	19	9	-	9
20	63	20	18	6	15
21	60	7	2	-	3
22	74	33	20	9	21
23	64	29	20	10	20
24 b	63	41	16	6	21
25	56	27	3	-	10
28	62	18	12	-	10
29	66	32	18	10	20
30	64	34	14	-	16
32	80	8	4	-	4
33	68	19	-	-	6
34	69	30	18	12	20
35	67	25	14	-	13
38	74	22	6	-	9
39	69	18	6	-	8
41	71	12	8	4	8
43	62	9	-	-	3
45	84	24	16	6	15
46	67	28	14	2	15
47	58	26	10	-	12
48	73	23	9	-	13
50	66	32	18	6	19
51	76	23	13	6	14
52	68	36	28	12	25
53	64	22	18	4	15
54	84	32	18	7	19
55	80	40	18	2	20
TOTAL:	2907	1038	510	144	564
Average:	69.2	24.5	12.1	3.4	13.2

TABLE 4.

Dose: 0. 20 I.U/2 kg. rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs. fall at end of 1st hour	Abs. fall at end of 2nd hour	Abs. fall at end of 3rd hour	Average abs. fall over 3 hours.
1	76	18	-	-	6
4	67	18	-	-	6
5	69	32	2	-	11
6	82	25	1	-	9
7	80	43	26	7	25
8	68	18	-	-	6
9	67	45	34	4	28
10	60	23	7	-	10
11	73	32	21	6	20
12	64	37	19	11	22
13	54	9	-	-	3
15	62	-	18	11	10
16	69	7	-	-	2
17	63	23	18	8	16
19	70	34	21	11	22
20	62	37	27	-	21
21	71	9	-	-	3
22	90	40	26	8	25
23	79	29	12	-	14
24	62	7	17	11	12
25	56	23	21	-	15
26	82	30	11	-	14
27	78	36	12	-	16
28	68	20	10	6	12
29	71	19	15	-	11
30	83	20	6	-	9
31	74	38	17	11	22
34	60	30	14	-	15
35	69	27	18	-	15
36	88	35	10	3	16
37	86	41	23	12	25
38	65	17	13	6	12
39	75	21	9	-	10
40	69	44	16	3	21
43	71	18	-	-	6
44	65	40	24	-	21
45	63	18	2	-	10
46	66	21	14	8	16
49	75	36	14	10	20
50	57	10	-	-	3
53	91	16	14	-	10
54	88	10	-	-	3
TOTAL:	2988	1062	522	136	573
Average:-	71.1	25.3	12.4	3.2	13.6

TABLE 5.

Dose: 0.25 I.U/2 kg.rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs.fall at end of 1st hour	Abs.fall at end of 2nd hour	Abs.fall at end of 3rd hour	Average abs.fall over 3 hours.
2	71	33	17	2	17
3	70	30	16	4	17
4	75	31	30	10	24
5	83	23	-	-	8
6	78	26	18	6	17
7	86	20	-	-	7
8	59	29	19	10	19
9	80	34	16	5	18
10	75	53	22	10	28
11	55	27	5	-	11
12	55	20	12	-	11
13	78	24	12	-	12
16	56	36	7	5	16
17	83	43	37	20	33
18	65	16	-	-	5
19	61	40	33	22	32
21	66	25	29	16	23
22	66	26	16	12	18
23	83	16	9	8	11
24	82	25	11	-	12
25	82	11	4	-	5
28	76	23	10	-	11
29	75	44	25	12	27
31	60	37	6	-	14
32	71	33	20	4	19
33	65	29	14	2	15
34	66	28	14	-	14
35	75	28	8	-	12
36	58	34	13	13	20
37	69	31	16	7	18
38	73	24	-	-	8
39	63	28	12	8	16
40	64	41	18	4	21
42	74	35	11	-	15
43	55	33	7	2	14
44	74	29	8	-	12
46	78	23	20	11	18
48	66	16	4	-	7
49	55	23	9	-	11
50	55	20	12	-	11
51	72	17	13	-	10
53	74	18	2	-	7
TOTAL:	2926	1182	555	194	642
Average:	69.7	28.1	13.2	4.5	15.3

TABLE 6.

Dose: 0.30 I.U/2 kg. rabbit.

Serial Number of Rabbit	Fasting blood sugar level	abs.fall at end of 1st hour	Abs.fall at end of 2nd hour	Abs.fall at end of 3rd hour	Average abs.fall over 3 hours.
1	67	27	13	5	15
2	86	35	24	-	20
4	71	43	13	-	19
5	61	17	-	-	6
7	71	33	16	7	19
8	74	34	18	8	20
9	64	33	3	-	12
10	79	28	20	-	16
12	82	46	28	8	27
13	87	32	9	-	14
14	78	39	24	7	23
18	78	18	-	-	6
19	59	5	13	-	6
20	72	37	16	5	19
21	68	13	11	1	8
22	61	13	9	-	7
23	77	33	21	11	22
24	68	34	17	-	17
25	72	27	-	-	9
26	72	35	18	7	20
27	83	25	13	10	16
28	80	37	10	-	17
29	79	33	23	5	20
31	56	23	16	-	13
32	75	34	17	7	19
33	75	17	15	-	10
34	64	28	14	4	15
35	69	35	22	4	20
36	83	38	21	-	20
38	56	12	6	-	6
39	69	19	9	-	9
40	68	30	23	3	19
41	84	30	11	-	14
42	68	31	16	9	19
43	65	30	17	4	17
44	58	18	4	-	7
46	74	80	24	11	22
47	65	20	5	-	8
48	76	28	12	8	16
49	61	29	7	-	12
52	71	36	20	4	20
53	69	34	20	4	19
TOTAL:	2997	1202	596	132	642
Average:	71.4	28.5	14.2	3. 1	15.3

TABLE 7.

Dose: 0.35 I.U/2 kg. rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs.fall at end of 1st hour	Abs.fall at end of 2nd hour	Abs.fall at end of 3rd hour	Average abs.fall over 3 hours.
1	76	44	33	20	32
3	67	39	25	16	27
5	79	10	15	-	8
6	68	38	18	4	20
7	71	18	5	-	8
9	69	30	4	-	11
10	85	38	28	-	22
11	80	27	23	-	17
12	81	33	18	-	17
13	80	28	-	-	9
14	60	25	10	8	15
15	75	44	16	4	21
16	70	31	11	-	14
21	68	42	41	23	35
22	73	39	29	12	27
23	60	33	6	-	13
26	71	31	19	2	17
27	79	18	4	-	7
28	75	35	15	11	20
29	83	42	17	2	20
30	79	8	4	-	4
31	77	25	-	-	8
32	81	21	14	-	12
33	74	47	36	8	30
34	78	32	14	-	15
35	66	42	38	19	33
36	77	21	6	-	9
37	65	36	24	15	25
38	73	33	18	4	18
39	77	22	2	-	8
40	66	31	13	5	16
41	69	25	-	-	8
42	83	32	16	3	16
43	69	36	9	-	15
44	56	24	16	-	13
45	67	18	10	-	9
47	58	24	9	-	11
48	79	28	16	2	15
49	78	19	4	-	8
51	71	37	30	8	25
52	73	32	18	7	19
53	68	19	17	-	12
TOTAL:	3056	1238	651	173	689
Average:	72.8	30	15.5	4.1	16.4

TABLE 8.

Dose: 0.40 I.U/2 kg. rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs. fall at end of 1st hour	Abs. fall at end of 2nd hr.	Abs. fall at end of 3rd hr.	Average abs.fall over 3 hours.	Abs.blood sugar level at end of 2nd hour.
1	72	17	-	-	6	72
2	81	29	19	3	17	62
3	78	32	21	4	19	57
4	79	37	29	8	25	50
6	55	24	18	4	15	37
7	78	48	27	9	28	51
8	72	37	12	-	16	60
9	79	18	2	-	7	77
11	79	24	18	12	17	64
13	60	23	37	21	27	23
14	56	19	14	-	11	42
15	86	31	9	-	13	77
16	74	17	13	-	10	61
18	92	35	16 n	10	20	76
20	61	18	18	3	13	43
21	69	31	13	-	15	56
22	56	26	21	5	17	35
25	69	39	36	5	27	33
26	79	43	20	-	21	59
29	71	46	21	-	22	50
31	70	8	-	-	3	70
33	72	18	3	-	7	69
34	78	17	8	-	8	70
35	57	27	20	7	18	37
38	73	19	-	-	6	73
39	89	37	18	8	21	71
40	71	44	23	3	23	48
41	76	18	12	4	11	64
42	84	30	10	3	14	74
43	80	32	18	4	18	62
44	70	33	14	-	16	56
45	78	30	23	3	19	55
46	59	23	16	-	13	43
47	76	44	23	7	25	53
48	57	27	21	4	17	36
49	76	49	27	8	28	49
50	72	40	12	-	17	60
51	80	27	18	15	20	62
53	78	20	23	3	15	55
54	69	43	36	8	29	33
55	62	23	18	3	15	44
56	59	38	22	21	27	37
TOTAL:	3032	1241	726	185	716	2306
Average	72.2	29.5	17.3	4.4	17.0	54.9

TABLE 9.

Dose: 0.45 I.U/2 kg. rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs.fall at end of 1st hour	Abs.fall at end of 2nd hr.	Abs. fall at end of 3rd hr.	Aver. abs.fall over 3 hours	Abs.blood sugar level at end of 2nd hr.
3	69	31	16	-	16	53
4	73	30	26	10	22	47
6	72	48	37	32	39	35
7	64	16	31	18	22	33
8	87	34	20	-	18	67
9	60	30	21	-	17	39
11	75	26	12	-	12	63
12	69	31	20	-	17	49
13	62	37	37	29	34	25
14	76	38	27	4	23	49
15	60	38	28	16	27	32
16	72	20	15	-	12	57
17	72	15	15	-	10	57
20	55	27	24	14	22	31
21	76	22	8	6	12	68
22	77	20	25	-	15	52
24	72	40	28	5	24	44
25	64	34	16	-	17	48
26	73	38	23	16	26	50
27	87	28	28	21	26	59
29	87	16	5	7	9	82
30	70	33	16	6	18	54
31	72	30	23	10	21	49
32	87	17	8	6	10	79
34	66	30	15	5	17	51
37	85	28	26	20	25	59
38	72	17	12	-	11	55
39	74	42	34	32	37	40
40	58	31	20	6	19	38
41	75	39	22	8	23	53
42	65	32	18	10	20	47
43	72	29	13	-	14	59
44	75	41	17	16	25	58
45	57	33	16	15	21	41
47	77	29	24	-	17	53
49	86	34	16	4	18	70
50	76	24	12	7	14	64
51	62	44	35	17	32	27
53	70	33	17	2	17	53
55	71	39	27	4	23	44
56	63	44	22	16	27	41
57	73	21	12	6	13	61
TOTAL:	3008	1287	872	368	842	2136
Average:	71.6	30.6	20.8	8.8	20.1	50.9

TABLE 10.

Dose: 0.50 I.U/2 kg. rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs.fall at end of 1st hour	Abs. fall at end of 2nd hour	Abs.fall at end of 3rd hour	Aver. abs. fall over 3 hrs.	Abs.blood sugar level at end of 2nd hr
1	65	37	18	11	22	47
2	66	36	7	7	17	59
3	67	39	31	18	29	36
4	68	39	25	--	21	43
6	62	42	40	24	35	22
7	72	36	39	17	31	33
8	78	16	-	31	16	78
9	76	43	13	-	19	63
10	65	29	28	-	19	37
11	73	47	28	7	27	45
12	69	32	17	5	18	52
13	79	9	22	17	16	57
15	81	21	22	17	20	59
16	74	16	45	17	26	29
17	63	28	25	-	18	38
18	81	37	27	20	28	54
20	76	16	15	-	10	61
21	75	42	38	18	33	37
22	70	25	25	16	22	45
24	69	34	43	30	36	26
25	79	19	43	39	34	36
26	64	34	18	9	20	46
27	77	28	14	-	14	63
30	73	31	29	8	23	44
32	78	45	40	6	30	38
34	80	37	17	3	19	63
35	65	32	16	-	16	49
37	74	38	41	11	30	33
38	72	36	26	13	25	46
39	75	21	10	-	10	65
41	66	39	36	7	27	30
42	68	24	46	29	33	22
43	64	26	18	10	18	46
44	67	32	17	9	19	50
46	69	24	36	-	20	33
47	80	35	35	9	26	45
48	78	23	19	6	16	59
49	68	29	14	5	16	54
50	62	30	20	11	20	42
51	61	34	44	16	31	17
53	75	28	24	-	17	51
54	71	19	11	-	10	60
TOTAL:	2995	1288	1082	446	937	1913
Average:	71.3	30.7	25.8	10.6	22.3	45.5

TABLE 11.

Dose: 0.55 I.U/2 kg. rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs. fall at end of 1st hour	Abs. fall at end of 2nd hour	Abs. fall at end of 3rd hour	Average absfall over 3 hours	Abs. blood sugar level at end of 2nd hr.
3	74	39	30	-	23	44
4	81	42	31	6	26	50
6	70	40	22	5	22	48
7	78	49	40	20	36	38
8	84	45	44	32	40	40
9	63	32	25	13	23	38
10	58	18	18	-	12	40
11	63	38	38	23	33	25
12	76	25	29	13	22	47
13	64	36	39	14	30	25
14	58	27	37	14	26	21
15	69	26	24	9	20	45
16	80	39	36	10	28	44
20	60	17	9	-	9	51
21	69	16	19	-	12	50
22	72	40	32	25	32	40
24	71	31	28	1	20	43
25	69	41	46	31	39	23
26	66	35	35	30	33	31
29	56	40	46	29	38	10
30	52	28	34	33	32	18
31	72	34	16	4	18	56
32	79	43	48	11	34	31
33	61	17	13	-	10	48
34	70	44	42	18	35	28
35	75	32	17	14	21	58
37	53	38	34	19	30	19
38	82	25	36	12	24	46
39	77	30	28	2	20	49
40	57	41	48	19	36	9
41	70	22	17	-	13	53
42	86	38	47	20	35	38
43	71	34	22	4	20	49
46	59	21	15	-	12	44
47	67	42	28	17	29	39
48	64	35	20	9	21	44
49	81	36	36	7	26	45
50	69	37	29	6	24	30
53	64	45	38	11	31	26
54	73	22	35	12	23	38
55	65	38	34	13	28	31
56	70	30	18	7	18	52
TOTAL:	2687	1408	1283	513	1064	1604
Average:	68.7	33.5	30.5	12.2	25.3	38.2

TABLE 12.

Dose: 0.60 I.U/2 kg.rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs.fall at end of 1st hour	Abs.fall at end of 2nd hour	Abs.fall at end of 3rd hour	Average abs.fall over 3 hrs.	Abs.blood sugar level at end of 2nd hour
1	74	35	20	-	18	54
2	75	45	27	9	27	48
3	77	50	36	15	34	41
4	68	25	22	13	20	46
6	62	20	20	-	13	42
7	76	37	25	-	24	51
8	61	42	35	20	32	26
9	79	49	49	36	45	30
10	74	24	29	-	18	45
11	77	34	41	30	34	36
12	60	29	24	12	22	36
13	75	51	47	31	43	28
14	84	59	59	51	55	25
15	70	32	30	15	26	40
17	72	39	31	9	26	41
18	64	25	41	27	31	23
20	68	25	34	3	21	34
22	61	29	21	9	20	40
24	92	53	53	20	42	39
25	63	15	29	23	22	34
26	69	28	31	15	25	38
27	67	35	28	-	21	39
29	85	45	44	19	36	41
31	73	31	21	9	20	52
32	70	37	30	9	26	40
33	74	30	38	16	28	36
35	72	39	29	14	27	43
37	64	24	35	9	23	29
38	61	31	14	-	15	47
40	76	55	49	58	54	27
41	91	50	40	31	40	51
42	76	38	35	23	32	41
43	63	18	35	11	21	28
44	76	37	20	10	22	56
46	66	41	38	17	32	28
47	67	34	38	24	32	29
49	61	39	44	13	32	17
50	73	28	45	8	27	28
51	78	50	46	30	42	32
53	62	31	17	-	16	45
54	76	38	37	30	35	39
56	76	32	46	45	41	30
TOTAL:	3008	1508	1433	714	1220	1575
Average:	71. 6	35.9	34.1	17.0	29.0	37.5

TABLE 13.

Dose: 0.65 I.U/2 kg.rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs.fall at end of 1st hour	Abs.fall at end of 2nd hour	Abs.fall at end of 3rd hour	Average abs.fall over 3 hours	Abs.blood sugar level at end of 2nd hr.
1	82	20	16	-	12	66
3	78	45	31	21	32	47
4	94	58	63	38	53	31
6	75	38	30	16	28	45
7	66	31	22	6	20	44
8	78	27	60	54	47	18
9	63	34	24	17	25	39
10	56	25	37	7	23	19
11	83	48	57	21	42	26
12	60	28	19	5	17	41
13	72	50	58	55	54	14
14	69	43	38	16	32	31
15	82	57	43	39	46	39
16	81	45	32	6	28	49
17	70	58	46	37	47	24
18	69	54	55	25	43	14
20	78	52	43	25	48	35
21	64	20	10	3	11	54
22	66	37	37	10	28	29
24	73	49	40	34	41	33
25	71	40	48	35	41	23
26	82	41	35	22	33	47
27	55	28	21	16	22	34
29	62	33	24	16	24	38
30	77	55	53	29	46	24
31	65	28	17	12	19	48
32	74	37	29	15	27	45
33	93	48	57	22	42	36
35	77	45	36	13	31	41
38	81	32	36	25	31	45
40	70	46	44	31	40	26
42	92	39	50	30	40	42
43	65	33	34	14	27	31
44	63	47	47	21	38	16
46	77	43	31	16	30	46
47	68	36	47	43	42	21
48	69	48	42	40	43	27
50	80	52	40	27	40	40
51	81	32	54	43	43	27
53	68	41	36	21	33	32
54	71	35	28	5	23	43
55	59	33	16	-	16	43
TOTAL:	3059	1691	1586	951	1408	1473
Average:	72.8	40.3	37.8	22.6	33.5	35.1

TABLE 14.

Dose: 0.70 I.U/2 kg.rabbit.

Serial Number of rabbit	Fasting blood sugar level	Abs.fall at end of 1st hour	Abs.fall at end of 2nd hour	Abs. fall at end of 3rd hour	Aver. abs. fall over 3 hrs.	Abs.blood sugar level at end of 2nd hour.
3	80	54	47	34	45	33
4	80	44	45	43	44	35
6	92	63	56	41	53	36
7	79	52	56	37	48	23
8	74	38	36	24	33	38
9	69	45	38	35	39	31
10	81	57	62	36	52	19
11	62	37	24	26	29	38
12	61	28	19	15	21	42
13	60	36	32	22	30	28
14	87	58	56	25	46	31
16	58	27	29	13	23	29
17	75	52	44	31	42	31
18	68	37	31	22	30	37
20	86	64	67	46	59	19
21	79	55	47	28	43	32
24	73	38	47	36	40	26
25	68	42	39	20	34	29
26	77	24	48	33	35	29
27	67	32	30	12	25	37
29	70	41	39	31	37	31
30	71	44	44	21	36	27
31	70	52	42	37	44	28
33	68	28	26	25	26	42
35	62	31	21	-	17	41
36	91	58	61	36	52	30
37	60	34	32	17	26	28
40	63	36	38	22	32	25
41	71	48	35	35	39	36
42	67	23	35	26	28	32
44	69	42	38	11	30	31
46	83	58	61	25	48	22
47	64	32	23	14	23	41
49	72	51	38	32	40	34
50	73	47	54	32	44	19
51	78	47	38	38	41	40
53	70	40	38	34	37	32
54	65	42	42	23	36	23
56	63	30	18	-	16	45
57	76	56	42	17	38	34
58	74	47	39	37	41	35
59	75	35	38	22	32	37
TOTAL:	3031	1806	1696	1114	1535	1336
Average:	72.2	43.0	40.4	26.5	36.5	31.8

TABLE 15.

Dose: 0.75 I.U/2 kg. rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs. fall at end of 1st hour	Abs. fall at end of 2nd hour	Abs. fall at end of 3rd hour	Average abs. fall at over 3 hours	Abs. blood sugar level at end of 2nd hour.
1	82	45	36	34	38	46
3	72	35	39	34	36	33
4	73	37	32	17	29	41
6	78	49	45	12	35	33
8	79	36	19	-	18	60
9	84	53	47	41	47	37
10	66	48	32	24	34	34
12	77	40	52	29	39	25
14	84	56	44	42	47	40
15	82	38	53	35	42	29
16	72	34	27	8	23	45
18	76	41	44	31	39	32
21	68	52	36	16	35	32
24	79	56	48	30	45	31
25	81	54	40	27	40	41
26	75	39	29	18	29	46
27	65	34	36	15	28	29
29	71	51	42	16	36	29
30	70	39	38	25	34	32
31	69	28	26	11	22	43
32	68	44	35	32	37	33
33	52	26	19	12	19	33
35	56	32	23	12	22	33
36	66	48	46	22	39	20
37	69	37	51	23	37	18
38	91	57	68	44	56	23
40	72	53	41	32	42	31
41	89	62	61	36	53	28
42	60	41	37	34	37	23
44	80	54	58	35	49	22
46	69	52	36	29	39	33
47	88	56	62	36	51	26
48	64	39	43	22	35	21
49	76	57	46	36	46	30
50	63	38	37	25	33	26
53	88	46	49	41	45	39
54	63	48	49	30	42	14
55	84	58	63	44	55	21
56	78	51	47	32	43	31
57	60	36	29	21	29	31
58	81	54	57	43	51	24
59	74	29	58	47	45	16
TOTAL:	3094	1883	1780	1153	1602	1315
Aver:	73.7	44.8	42.4	27.4	38.1	31.3

TABLE 16.

Dose: 0.80 I.U/2 kg. rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs.fall at end of 1st hour	Abs.fall at end of 2nd hour	Abs.fall at end of 3rd hour	Average abs.fall over 3 hours	Abs.blood sugar level at end of 2nd hour.
3	73	44	51	43	46	22
6	65	50	46	6	34	19
7	89	56	55	62	58	34
8	84	52	46	49	49	38
10	78	37	29	17	28	49
12	68	26	36	26	29	32
13	92	67	76	44	62	16
14	59	43	43	22	36	16
16	62	36	33	33	34	29
19	77	50	45	28	41	32
20	71	32	48	49	43	23
21	66	43	40	21	35	26
23	76	35	17	-	17	59
24	85	52	63	44	53	22
25	80	54	45	38	46	35
26	64	43	44	22	36	20
28	75	56	49	21	42	26
29	73	37	39	29	35	34
31	69	36	34	22	31	35
33	79	52	63	44	53	16
35	81	62	61	40	54	20
36	66	34	26	6	22	40
37	69	25	39	47	37	30
39	74	52	38	31	40	36
41	78	46	59	26	44	19
42	58	31	29	24	28	29
44	81	52	43	31	42	38
46	65	31	38	30	33	27
47	76	48	48	27	41	28
48	74	38	45	25	36	29
49	69	41	40	22	34	29
51	70	40	19	25	28	51
53	70	43	46	28	39	24
55	73	56	58	33	49	15
56	63	42	41	31	38	22
58	68	35	48	23	36	20
59	77	58	54	43	52	23
61	84	52	63	47	54	21
62	72	50	48	31	43	24
64	60	42	39	21	34	21
65	75	53	46	40	46	29
66	70	44	41	34	40	29
TOTAL:	3058	1876	1871	1285	1678	1189
Average:	72.8	44.6	44.5	30.5	40.	28.3

TABLE 17.

Dose: 0.85 I.U/2 kg. rabbit.

Serial Number of Rabbit	Fasting blood sugar level	Abs.fall at end of 1st hour	Abs.fall at end of 2nd hour	Abs.fall at end of 3rd hour	Average abs.fall over 3 hours	Abs.blood sugar level at end of 2nd hr
3	65	44	52	18	38	13
4	68	32	17	-	16	51
6	79	38	56	46	47	23
8	74	53	45	28	42	29
9	81	48	20	-	23	61
11	66	36	47	34	39	19
12	72	54	50	8	37	22
14	70	13	46	28	29	24
16	78	53	51	35	46	27
18	85	58	67	52	59	18
19	69	34	51	38	41	18
21	73	42	40	32	38	33
23	78	58	47	30	45	31
24	62	13	38	30	27	24
26	67	36	36	39	37	31
27	77	47	38	44	43	39
28	80	-	34	30	21	46
30	64	44	41	21	35	23
31	88	59	68	62	63	20
33	58	53	30	15	26	28
34	76	46	49	43	46	27
36	56	34	7	-	14	49
37	66	37	46	32	38	20
39	72	53	41	34	43	31
40	70	50	41	30	40	29
41	89	51	65	52	56	24
43	68	47	38	21	35	30
46	77	47	53	30	43	24
47	73	28	43	52	41	30
49	75	52	33	33	39	42
51	69	51	50	13	38	19
52	72	29	27	22	26	45
53	80	43	53	35	44	27
55	79	58	63	47	56	16
56	78	42	55	51	49	23
58	71	38	47	44	43	24
59	65	50	38	29	39	27
61	68	46	46	41	44	22
63	71	40	39	29	36	32
64	73	52	51	34	46	22
65	69	46	52	45	48	17
67	74	54	57	42	51	17
TOTAL:	3045	1789	1868	1349	1661	1176
Average:	72.5	42.5	44.5	32.1	39.5	28.0

TABLE 18.

SUMMARY OF TABLES 1- 17.

Group.	Dose I.U/2 kg. rabbit	Average fasting blood sugar level	Average absolute fall at end of 1st hr.	Average absolute fall at end of 2nd hr.	Average absolute blood sugar level at end of 2nd hour	Average absolute fall at end of 3rd hour	Average absolute fall over 3 hrs.	Average percentage fall over 3 hours
1	0.005	69.4	5.5	-	69.4	-	1.8	2.6
2	0.10	72.0	11.5	1.5	70.5	-	4.4	6.1
3	0.15	69.2	24.5	12.1	57.1	3.4	13.2	19.1
4	0.20	71.1	25.3	12.4	58.7	3.2	13.6	19.1
5	0.25	69.7	28.1	13.2	56.5	4.5	15.3	21.9
6	0.30	71.4	28.5	14.2	57.2	3.1	15.3	21.4
7	0.35	72.8	30	15.5	57.3	4.1	16.4	22.5
8	0.40	72.2	29.5	17.3	54.9	4.4	17.0	23.5
9	0.45	71.6	30.6	20.8	50.8	8.8	20.1	28.6
10	0.50	71.3	30.7	25.8	45.5	10.6	22.3	31.3
11	0.55	68.7	33.5	30.5	38.2	12.2	25.3	36.8
12	0.60	71.6	35.9	34.1	37.5	17.0	29.0	40.5
13	0.65	72.8	40.3	37.8	35.0	22.6	33.5	46.0
14	0.70	72.2	43.0	40.4	31.8	26.5	36.5	50.6
15	0.75	73.7	44.8	42.4	31.3	27.4	38.1	51.7
16	0.80	72.8	44.6	44.5	28.3	30.5	40	54.9
17	0.85	72.5	42.5	44.5	28.0	32.1	39.5	54.4

TOTAL: 1215.0

Average: 71.5

It will be noticed that the average fasting blood sugar levels for the 17 groups are very closely spaced - too close for correction of the results by application of a correction figure, as indicated by Marks and Hemmingsen (13) and de Jongh, Lens and Spanhoff (28). However, it was felt that even such slight differences affect the values in the succeeding columns, especially as far as the average absolute blood sugar level at the end of the second hour (column 6, Table 18) is concerned. It was, therefore, decided to obtain the average of the average fasting blood sugar levels (i.e., 71.5) and to express the succeeding results in terms of this common fasting blood sugar level, the corrected results being given in Table 19:

TABLE 19.

SUMMARY OF TABLES 1-17,
VALUES CORRECTED TO A MEAN FASTING BLOOD SUGAR LEVEL OF 71.5.

Group	Dose I.U/2 kg. rabbit	Average absolute fall at end of 2nd hour	Average absolute blood sugar level at end of 2nd hr.	Average absolute fall over 3 hours.
1	0.05	-	71.5	1.9
2	0.10	1. 5	70.0	4.4
3	0.15	12.5	59.0	13.7
4	0.20	12.8	59.0	13.7
5	0.25	13.5	58.0	15.7
6	0.30	14.2	57.3	15.3
7	0.35	15.2	56.3	16.1
8	0.40	17.1	54.4	16.8
9	0.45	20.8	50.7	20.5
10	0.50	25.9	45.6	22.4
11	0.55	31.7	39.8	26.3
12	0.60	34.0	37.5	29.0
13	0.65	37.1	34.4	32.9
14	0.70	40.0	31.5	36.1
15	0.75	41.0	30.4	37.2
16	0.80	43.7	27.8	39.3
17	0.85	43.9	27.6	38.9

/(d)

(d) Discussion.

(i) General Considerations:-

The fasting blood sugar values of rabbits, as obtained by various workers from time to time, show great variations. Scott, Ford and Eadie (quoted from 191) found a mean blood sugar of 0.117 per cent, with variations from 0.068 to 0.177 per cent. In Murlin's laboratory (loc.cit) the mean was found to be 0.105 with variations from 0.071 to 0.143, whereas Grevenstuk and Laqueur (loc.cit) obtained a mean value of 0.138. Clough et al (22), from 764 readings representing 64 animals, reported a fasting value of 0.113 per cent and a standard deviation from the mean of 17.8 mg. In this series of 714 readings an average value of 0.071 per cent, with variations from 0.052 to 0.093 per cent has been obtained. The standard deviation of the whole series was found to be 8.84. These apparent discrepancies in the fasting values obtained by different workers are due, as has already been indicated, to the methods of sugar estimation, the various older ones having been compared and discussed by Herbert et al.(23). The low average fasting value obtained in this series (0.071 per cent, as compared to 0.117, 0.105, 0.138, 0.113 per cent of other workers) is the result of the method of sugar determination (20), which estimates glucose of the plasma alone and excludes the nitrogenous reducing compounds of the corpuscles by the use of isotonic sodium sulphate. Rabinowitch (24) had postulated that non-glucose reducing substances are responsible for 0.025 per cent and have a value of 16.31 mg.per 100 ml. of the reducing value of the blood (page 3).

Ernst and Foster (172) claim that non-glucose reducing substances have a value of from 0.024 to 0.049 per cent. Viewed in the light of these findings, the average result obtained in this series compares favourably

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with those of other workers. When the earlier observers (4), therefore, postulated 0.045 per cent as the convulsive level in rabbits, most of this figure, as was shown by Hiller et al (25) and Dotti (173), represented non-glucose reducing substances. With this series it was consistently found that the blood glucose in the rabbit drops to an appreciably low level (0.005 to 0.010 per cent) before signs of convulsions become evident.

The individual variations in fasting value ($\pm$0.052 - $\pm$0.093), as seen in each of the preceding Tables 1 - 17, cannot be accounted for. These are well-known to all workers in this field and occur, notwithstanding the utmost care as regards feeding of the animals, period of fasting, etc. The inconsistency of this value in a specific animal is illustrated by the following figures representing the fasting blood sugar of rabbit No.9 on 15 different occasions: 0.071, 0.066, 0.067, 0.080, 0.064, 0.069, 0.079, 0.060, 0.076, 0.063, 0.079, 0.063, 0.069, 0.084 and 0.081. These are probably the result of daily variations due to external and unidentified internal physiological factors. However, an important observation that was made in this respect is that, on a particular day, rabbits that had received similar treatment, almost invariably show similar fasting values. Values like the following were obtained more as a rule than exception:-

Day X : Rabbit 24 - 0.068 per cent,
Rabbit 25 - 0.066 per cent
Rabbit 27 - 0.069 per cent.

Day Y : Rabbit 24 - 0.080 per cent,
" 25 - 0.078 " "
" 27 - 0.084 " "

Values such as, e.g., 0.058, 0.072, 0.089 per cent on one day for 3 similarly-treated animals were hardly ever encountered. When this was the case, the cause could, in

/most

most instances, be traced to some other factor, such as unequal fasting periods, excitеability of rabbits, faulty determination, etc. The observation, substantiated by Lacey (192), that on a specific day the fasting blood sugar values of a group of similarly-treated animals usually vary within narrow limits, forms an important basis of the method of activity evaluation to be described later.

The fact that the average values (fasting) for the different groups vary within such narrow limits, is probably due to the pattern of procedure followed with injection of the various groups (page 27).

The next point requiring consideration, is the effect of insulin with reference to the time period. In the assay of insulin, the study of the blood sugar fall over a period of 5 hours was originally adopted as a result of the observations (14, 191) that, after this period, the blood sugar level had in most cases returned to normal. That this is not the case, is amply confirmed by studying the results of insulin assays as published and by the work of Thorp (40). It was, therefore, thought that, with the reduction of the doses ordinarily used in assays, the time period of five hours could safely be reduced to three. The results quoted in the preceding tables show that, with the exception of the small doses (0.05 - $\pm$0.30 I.U/2 kg), the blood sugar does not return to its normal value within the first three hours. However, it is clear from the quoted results that the duration and degree of response show a marked parallelism, and, in order to obviate determinations necessary for studying the duration of the response, a study of the degree, as exhibited at different time intervals over a definite time period, is apparently equally effective. That this response is graded for each period tested over a wide range of doses, is clearly

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shown by the results, and will be referred to more in detail presently.

To determine accurately the lowest point to which the blood sugar falls after insulin administration is wellnightimpossible. How closely this lowest point can be approximated will depend on the frequency of the bleedings, which, for practical reasons, cannot be too often. However, a comparative estimate is obtained by bleeding at accurately executed post-injection periods. Employing this method, a study of the average falls after 1, 2 and 3 hours shows that, with the entire dosage range tested, a maximum fall is obtained within the first hour, which is then maintained according to the amount of dose. In the lower ranges (0.05 - ± 0.45 I.U/2 kg.), the average fall at the end of the second hour is appreciably less than that of the first. For the remaining doses (0.50 - 0.85 I.U/2 kg.) the maximum fall of the first hour is maintained during the second towards the third hour. This indicates that increasing dosage first affects the maximum fall and, secondly, the duration of the fall. It is well-known that, with convulsive doses, convulsions hardly ever set in within the first hour after injection, but more often round about the second towards the third hour. This probably indicates that the maximum fall had not been reached until that time, but also that the duration of the fall is an important causative factor in producing convulsions. It appears that the normal organism has some protective mechanism against a transient fall of the blood sugar, but that this resistance is overcome by a maintained low level.

Marks (14), studying the dose-response ratio over a period of 5 hours, concludes, inter alia, that "with larger doses the hypoglycaemic effect reaches a limit, above which there is practically no further increase,

/however

however large the dose? This "limit" is certainly conditioned by the time period allowed, and in this series (with smaller doses over 3 hours only) the limit seemed to have been reached by the 0.80, 0.85 I.U/2 kg. doses (due to the maintained effect). Similarly, in Marks's experiment, the limit was reached by the maintained effect of the larger doses over a longer period. If, in the same experiment, blood samples had been taken at 6, 7, 8 and 9 hours after injection (instead of discontinuing at 5), and doses gradually increased (provided convulsions did not occur), his dose-response ratio curve would probably not have shown the horizontal line for doses over 1 - 1.5 unit per kilo.

The effect of small doses of insulin has received attention from Scott and Dotti (195). With this is bound up the question whether an insulin dose must be of specific amount before it affects the blood sugar, or whether added insulin, however small, does have a measurable effect. The statement has been made that light doses have no effect or that there is an "all or none" behaviour. Scott and Dotti (loc.cit). published data relating to the response after injection of 1/16th - 1 unit per kilogram over 3½ hours. They found a blood sugar decrease proportional to the dosage to 1/16 unit per kilo and, by extrapolation, calculated a percentage reduction of 10 per cent for a dose of 1/32 unit per kg. Whether doses smaller than this cause a relatively smaller reduction of the blood sugar, or whether the curve shows a similar flattening in the extreme low reaches as is the case with high doses, is difficult to determine. This is due to two factors: (i) The precision of the best methods for the determination of the blood sugar does not permit differentiation of sugar concentrations after administration of very small doses, and (ii) the effect of small doses is probably so short-lived that, in order to determine it, blood should

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be withdrawn at very short intervals after injection. The authors suggest that $\frac{1}{8}$ and $\frac{1}{4}$ unit per kg. are two very satisfactory dosages with which to work, and conclude that "the failure of our technique to demonstrate the extremely small changes in blood sugar level which are to be expected to result from very small doses of insulin, can hardly be interpreted as indicating that they do not exist. As insulin continues to have the expected effect as far down the curve as it can be followed technically, it is difficult to justify assumptions that it acts differently beyond this point. We are, therefore, unable to interpret our data as giving any support to the hypothesis that light doses of insulin have no effect, or that insulin in any manner acts according to an 'all or none' principle."

It should be emphasized that the measurement of effect, as was done in the experiments of Scott and Dotti and also in this series, entails the measurement of degree over a specific time period. As the latter is changed, so will the effect vary. For instance, if the effect of a 0.5 unit/2 kg.dose over a period of 3 hours is given as an absolute blood sugar reduction of 22.0, the same effect measured over a period of 5 hours, would be appreciably less, depending on how soon the hypoglycaemic effect is worn off and the blood sugar again reaches its pre-injection level. If, in the above example, this happens at the end of the third hour and the determination made over 5 hours, then the effect of 22.0 (quoted above) will be reduced to only 13.0. With this fact reserved, it can be stated that, measured over a period of 3 hours, doses of 0.05 and 0.10 units per 2 kg.have no hypoglycaemic effect (see Tables 1 and 2) and that the dosage-response curve is similarly flattened out in its lower reaches than in the upper ones (see Figure 1 and Table 18).

The limit of precision of the method used for blood sugar determinations was calculated at 9 per cent. Consequently doses of 0.05 and 0.10 units must be considered as having no effect over 3 hours, with the first bleeding taking place at 1 hour post-injection. As it is, 62 per cent of the animals still show a measurable reduction after 1 hour with a dose of 0.05 units and 74 per cent with a dose of 0.10 units. If only those reductions exceeding the experimental error of 9 per cent are considered, then the respective figures would be 24 per cent and 50 per cent. Added to this is the possibility that, in many rabbits showing no response after 1 hour, the blood sugar had probably already returned to its pre-injection value at the time of bleeding. If, with very small doses, the effect was measured over a shorter period, say, 1 hour, the bleeding schedule adjusted to say, 15, 30, 45 and 60 minutes, and methods of sugar determination sufficiently accurate, it is probable that extremely small doses of the hormone will show a well-graded measurable effect.

The flattening of the dosage-response curve in its upper reaches (Figure 1) has already been argued to be due to the maintained effect over 3 hours. If, in Table 15, 16 and 17, the response was measured over 5 instead of over 3 hours, then the flattening of the curve would undoubtedly not have occurred. The flattening in the lower reaches (i.e., with doses of 0.15, 0.20, 0.25 and 0. 30 units per 2 kg) still needs explanation. Tables 3, 4, 5 and 6 show that the absolute reduction over 3 hours is 13.2, 13.6, 15.3, 15.3 respectively. If, however, the reduction is measured over 2 hours, the respective figures would be 18.3, 18.9, 20.7 and 21.4, thus indicating a more distinct proportionabity. Similarly,

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if the response had been measured over 1½ hours with a bleeding schedule of ½, 1 and 1½ hours, it is probable that the proportionality would have been more pronounced still. As Macleod (191) and Scott and Dotti (195) pointed out, the velocity of change varies in different animals. Drawing of blood at predetermined times (1, 2, 3 hours, etc.) entails the measurement of decreasing (towards the minimum) sugar values in some animals and increasing (returning towards the fasting level) in others. It is, therefore, possible that, measuring the effect of the quoted dosage range over 3 hours with a bleeding schedule of 1, 2 and 3 hours, is the causative factor in the flattening of the curve for the lower levels. Scott and Dotti point out that "by strict theory the time of drawing the blood samples should be so chosen for each dose that the maximum initial velocity of change will be measured. The conclusion, therefore, seems feasible that the dosage-response curve shows proportionality over a much wider range of doses than is generally accepted, the flattening of the curve in its upper limits being due to the masking effect of too short a period of measurement, and in its lower limits to the masking effect of too long a period of measurement. It is suggested that with the higher and lower doses, not only the time period but also the bleeding schedule should be adapted to the dosage.

(ii) Considerations specific to the dose-response curve.

It will be seen that, in each group, the fasting value varies from ± 0.052 - ± 0.090 per cent. The question, therefore, arose whether the resulting fall values should

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be corrected for differences in fasting values by application of a correction factor as advocated by Marks and Hemmingsen (13) and de Jongh, Lens and Spanhoff (28). This was tried out by correcting the average absolute fall over 3 hours, as presented in Table 10, by the regression coefficient of Marks, viz: 0.032. The result obtained varied so slightly from the original (22.4 as compared to 22.3) that it was decided to discard correction of the fall values in favour of a general correction of the average values, as shown in Table 19, and to be discussed presently.

The effects of graded doses of insulin can be studied from the summary in Table 18 or the summary in Table 19 showing corrected values. From both of these it is clear that the blood sugar value decreases in approximate proportion with the increase of dosage. Column 8, Table 18, represents the average absolute reduction over a period of three hours following the injection of doses of insulin from 0.05 - 0.85 I.U/2 kg. and varying with 0.05 units. In column 9 the average absolute reduction has been converted to the average percentage reduction. With the lower doses (0.05 - 0.25) no clear proportionality can be seen between dosage and effect. This is probably due, as has been explained, to technical difficulty and experimental error in determining slight responses. Similarly, with the higher doses (0.75 - 0. 85) the proportionality is masked by the maintained fall over periods longer than 3 hours. In between these two limits is a range of doses (0.30 - 0.75) where the hypoglycaemic effect (expressed as either absolute or percentage reduction) is approximately proportional to the dose. Using the data of column 8, Table 18, correcting them to a mean fasting blood sugar level of 71.5 (column 5, Table 19) and plotting hypoglycaemic effect against dosage, the

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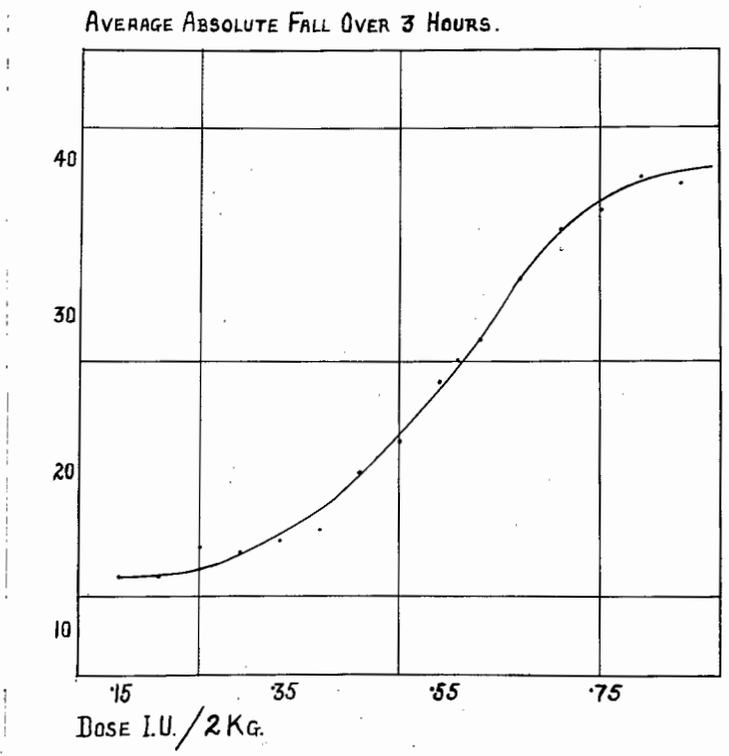
curve, shown in Figure 1, was constructed. The flattening at the higher and lower levels and the proportional effect at the mid-levels are clearly illustrated.

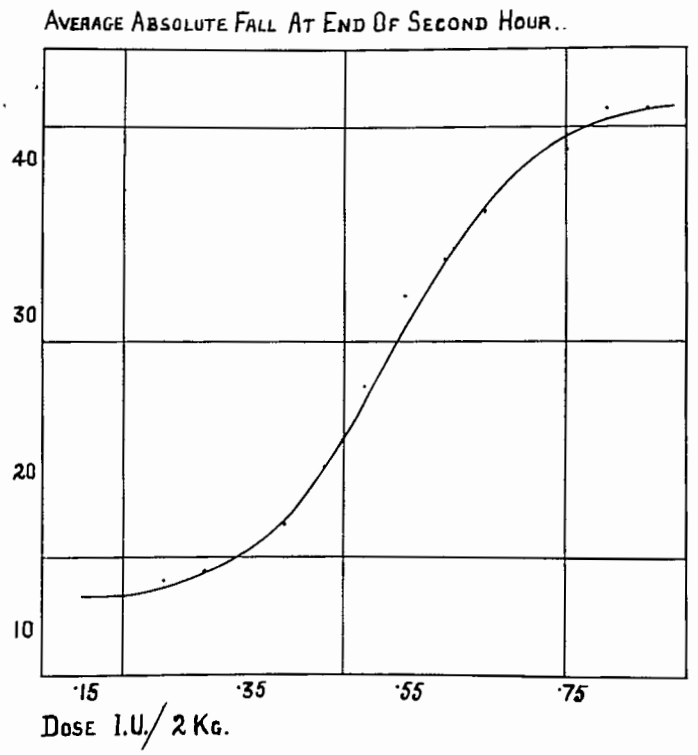
Figure 1. Average absolute fall over 3 hours.



However, this proportionality is not seen in column 8, Table 18, only. Columns 4, 5 and 7 (average absolute fall at the end of the first, second and third hours, respectively) show a similar proportionality. In columns 4 and 7 (average absolute fall at the end of the first and third hours) the useful range is much shorter than that of column 8 and, therefore, less suitable for practical application. They were, therefore, discarded. In contrast to this, the data of column 5 (average absolute fall at the end of the second hour) show a useful range which compares very favourably with that of column 8. These data were subsequently corrected to a mean fasting value of 71.5 (column 3, Table 19) and used to construct the curve shown in Figure 2.

/Figure 2.

Figure 2. Average absolute fall at end of 2nd hour.



Comparison of curves 1 and 2 immediately shows that each of these apparently represents the hypoglycaemic effect of varying doses of insulin over 3 hours equally effectively. The overall application of this observation is that the dosage-response ratio can be determined by initial bleeding, injection and subsequent bleeding after 1, 2 and 3 hours, or by initial bleeding, injection and subsequent bleeding after two hours only. It is obvious that, practically considered, the latter procedure is preferable. However, it is clear that similarly-graded average responses, with consequent similar dosage-response curves (c.f.Figures 1 and 2) are no true measure of an equal effficiency of the two procedures. For instance, if an absolute fall of 35 over 3 hours is the resultant mean of falls of 26, 38 and 41, and a similar absolute fall of 35 at the end of the second hour, the resultant mean of falls of 17, 21 and 67, it is obvious that, for practical application, the former procedure would be much more reliable than the latter. In order, then, to test the reliability of the two procedures, the following method was adopted: For each dose

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in the useful range (0.40 - 0. 70 units/2 kg.- see page 68) the standard deviation of the series of responses was determined and for each standard deviation the coefficient of variation calculated, the results being listed in Table 20:-

TABLE 20.

Standard Deviations and Co-efficients of Variation of Absolute Reductions of the Blood Sugar after 2 and over 3 Hours.

Group	Dose I.U/ 2 kg. rabbit	Average absolute fall at end of 2nd hr.		Average absolute fall over 3 hours.	
		Standard Deviation	Co-efficient of variation (per cent)	Standard deviation	Coefficient variation (per cent)
8	0.40	8.9	51.7	6.8	40.0
9	0.45	7.7	37.1	7.1	35.2
10	0.50	11.6	45.1	6.7	30.0
11	0.55	10.4	34.1	8.2	32.5
12	0.60	10.4	30.5	9.9	34.0
13	0.65	13.4	35.4	11.0	33.0
14	0.70	11.8	29.3	9.8	26.8
		TOTAL:-	263.2		231.5
		Average	37.6		33.1

These results show an average coefficient of variation of 37.6 per cent for the average absolute fall at the end of the second hour, and 33.1 per cent for the average absolute fall over 3 hours. This indicates a slight advantage of the latter over the former procedure.

Regarding the optimum time interval, Scott and Dotti (195) point out that "it is difficult to see how the use of the minimum blood sugar value as the criterion may be made practical, for it cannot be determined by averages, nor located by a single determination, while a series of determinations is impractical, both physiologically and

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technically. The question then arises as to whether there may not be some point on the curve other than the minimum which could be established by averages. The practical definition of such a point must be in terms of time rather than of blood sugar level. The selection of a definite interval after the administration of the insulin, at which the sugar level shall be determined, amounts to a determination of the rate of blood sugar change rather than a measure of the absolute change." These authors find that, if the blood samples are drawn 30 minutes after the injection of insulin, the relative drop in blood sugar is proportional to the logarithm of the dose through a considerable portion of the practical dose range.

Due to the bleeding schedule (1, 2 and 3 hours) in this series, this claim can neither be substantiated nor rejected. It is clear, however, that determination of the blood sugar level only 30 minutes after injection is a measurement of degree in terms of rate of effect, which has been shown, for the lower doses, to reach the minimum value at any time within one hour. On the other hand, determination of the blood sugar level 2 hours post injection is more a measurement of degree in terms of duration of effect, which, as can be seen from the data and constructed curves, shows a similar proportionality as that claimed by Scott and Dotti for 30 minutes post injection. It is generally agreed, and substantiated by the data in the preceding tables, that with the lower doses the rate of change from initial to minimum level is a quick process, usually completed within one hour or soon after injection. On the other hand, return of the blood sugar from the minimum level to the initial is a gradual process

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requiring, even with small doses, two or more hours for completion. On account of this, determination of the sugar level while gradually returning to normal, seems preferable to determining it while rapidly decreasing to the minimum.

In view of the above findings, it was next considered whether the calculations could not be based on the absolute reduced blood sugar level at the end of the second hour instead of on the average absolute reduction. If this were possible, the initial bleeding could be eliminated and the dose-response ratio determined by a single bleeding two hours after injection. The average absolute blood sugar levels two hours after injection (column 6, Table 18) were, therefore, studied and the data observed to indicate a proportionality. However, it was felt that, due to differences in fasting values (column 3, Table 18) the data in columns 5, 6 and 8 should be expressed in terms of a mean fasting value. For this, the average of the average fasting blood sugar levels was calculated (c.f 71.5 in column 3, Table 18) and the average absolute fall at the end of the second hour, the average absolute level at the end of the second hour and the average absolute fall over 3 hours corrected to this by way of simple proportion, the results being given in columns 3, 4 and 5, Table 19.

Regarding column 4, it is clear that, with this procedure, the proportionality between dose and absolute blood sugar level two hours after injection, has been considerably enhanced. From these data, the curve shown in Figure 3, plotting absolute blood sugar level against the dose, was constructed. The similarity of this curve to those shown in Figures 1 and 2 is apparent. It was tested for reliability in the same way as the previous two, the combined results being given in Table 21.

/Figure 3.

Figure 3. Average absolute blood sugar level at end of 2nd hour.

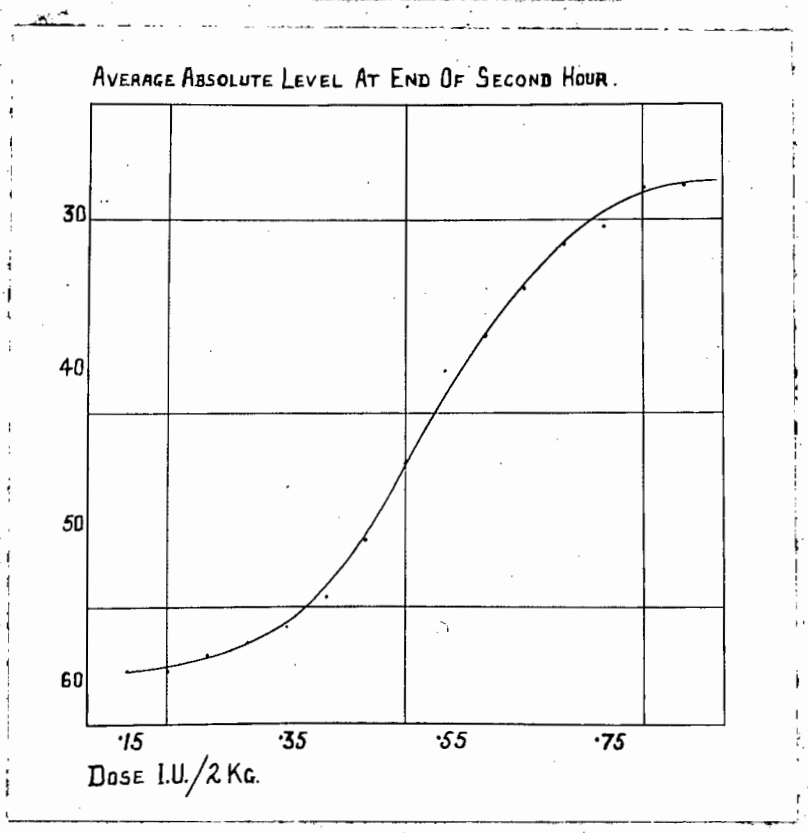

TABLE 21.

Standard Deviations and Coefficients of Variation of Absolute Reductions of the Blood Sugar after 2 and over 3 hours and of the Absolute Level of the Blood Sugar after 2 Hours.

Group	Dose I.U/2 kg. rabbit	Average absolute fall at end of 2nd hour		Average absolute fall over 3 hours.		Average absolute blood sugar level end of 2nd hour	
		Standard deviation -	Coefficient of variation (percent)	Standard deviation	Coefficient of variation (percent)	Standard deviation	Coefficient of variation (Percent)
8	0.40	8.9	51.7	6.8	40.0	13.9	25.4
9	0.45	7.7	37.1	7.1	35.2	12.7	25.0
10	0.50	11.6	45.1	6.7	30.0	13.3	29.3
11	0.55	10.4	34.1	8.2	32.5	12.1	31.7
12	0.60	10.4	30.5	9.9	34.0	9.0	24.0
13	0.65	13.4	35.4	11.0	33.0	11.5	32. 8
14	0.70	11.8	29.3	9.8	26.8	6.5	20.4
		TOTAL:	263.2		231.5		188.6
		Average:	37.6		33.1		26.9

The low coefficient of variation in the last column (26.9 per cent) not only shows that this procedure is as reliable as the other two, but also that it is more reliable. This observation, therefore, furnishes evidence in favour of those methods of determining insulin activity based on the absolute level of the blood sugar, rather than those based on the fall of the blood sugar, either absolute or percentage.

The three dosage-response curves and the results obtained by application of tests for reliability both indicate that the dosage-response in rabbits, after injection of suitable insulin doses, can be determined satisfactorily by any one of the following three procedures:-

(i) Initial bleeding, injection and post-injection bleeding at 1, 2 and 3 hours. The hypoglycaemic effect can be expressed either as the average absolute fall or the average percentage fall over 3 hours;

(ii) Initial bleeding, injection and post-injection bleeding at 2 hours. The hypoglycaemic effect can similarly be expressed as the average absolute fall or average percentage fall at the end of the second hour;

(iii) No initial bleeding, injection and post-injection bleeding at 2 hours. The hypoglycaemic effect is expressed in terms of the average absolute blood sugar level at the end of two hours.

Practical considerations clearly favour the latter procedure. An important advantage is the exclusion of the fasting blood sugar value. As previously indicated, Young and Romans (29) and Pugsley and Rampton (30) have found that, in both the subcutaneous and intravenous methods, the initial sugar value may be excluded from the calculations without affecting the precision of the assay.

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The basis for this statement is that, in the two groups of rabbits, one of which receives the standard and the other the unknown, initial differences are counterbalanced. Added to this are the observations of Marks and Hemmingsen (13) and de Jongh, Lens and Spanhoff (28) that both the absolute and percentage fall increase with increasing initial value. Laqueur (31) also ignored the initial blood sugar value and merely observed the lowest point to which the blood sugar fell during the test. Moreover, it was observed during the present tests that, on a specific day, the blood sugar values of a similarly treated group vary within narrow limits. This was also substantiated by Lacey (192). In view of this evidence, it seems justifiable, for comparative purposes (i.e., standard against unknown) to omit the initial sugar value. Whether the same procedure can be adopted in a simple dosage-response study (where no comparison between the effect of standard and unknown is made), is perhaps less substantiated by experimental evidence. However, the results of Table 21 clearly prove that, provided a sufficient number of animals are used, over the normal variations of fasting values encountered, the initial blood sugar may be omitted, and calculations based on the absolute level of the blood sugar at some stage after injection.

The validity of such a procedure was further tested in the following way: The data in columns 5, 3 and 4, Table 19, were used to construct the dosage-response lines shown in Figures 4, 5 and 6, where the average absolute fall over 3 hours, the average absolute fall at the end of the second hour and the average absolute blood sugar level at the end of the second hour, respectively, have been plotted against the logarithm of the dose. Previous workers have established the fact that, for part of the dosage range,

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the effect is a logarithmic function of the dose, i.e, the dose-effect ratio plots as a straight line. In each of figures 4, 5 and 6 it is seen that the points representing the effects of doses 0.40 → 0.70 unit lie in an almost straight line. In the respective cases the straight lines were fitted to the average absolute fall over 3 hours, to the average absolute fall at the end of the second hour and to the average absolute level at the end of the second hour against the log (dose x 100) by the method of least squares. The positions of the points with regard to the straight lines are such as to indicate equal reliability for the three procedures. An additional observation in this respect is that this linear relationship is not specific within a single dosage range only, but can be extended to other ranges with correspondingly changed bleeding schedules.

Figure 4. Average absolute fall over 3 hours.

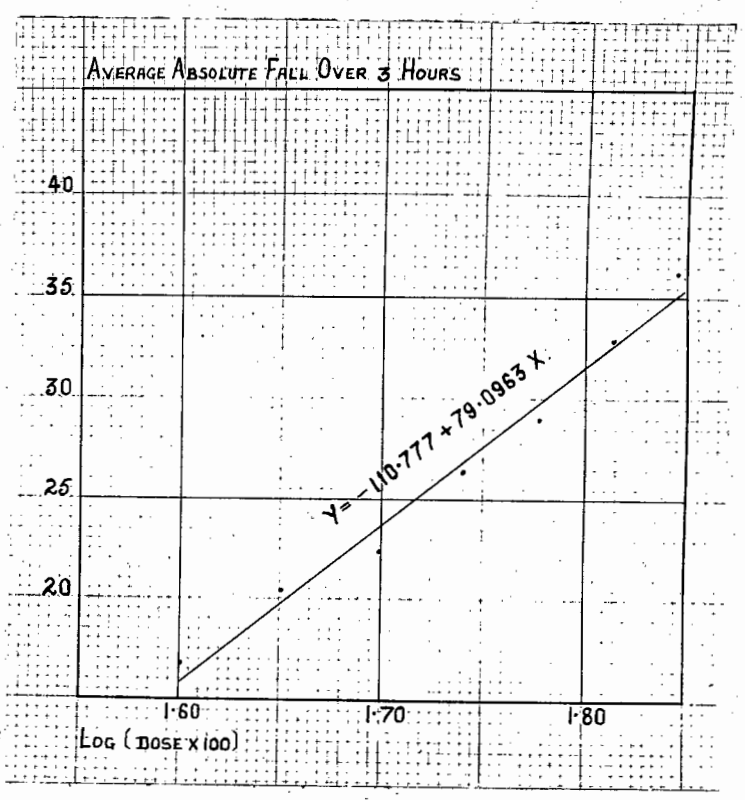


Figure 5. Average absolute fall at end of 2nd hour.

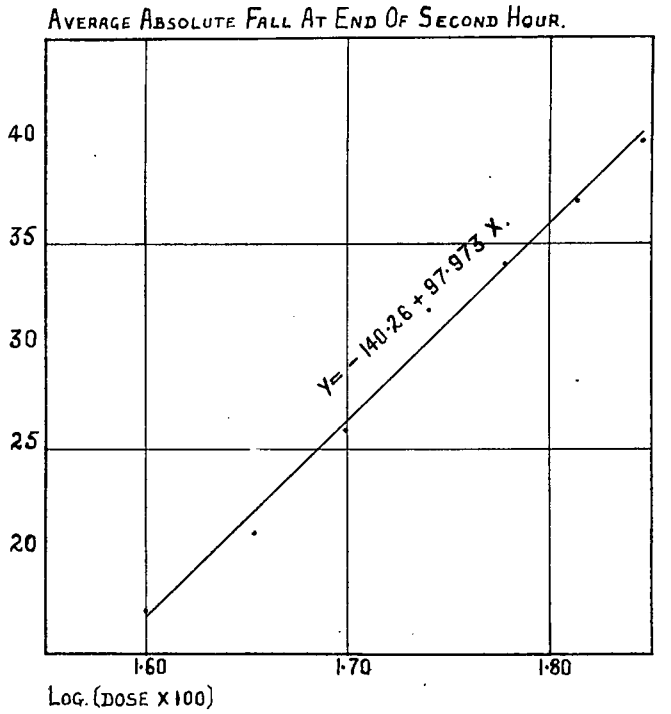
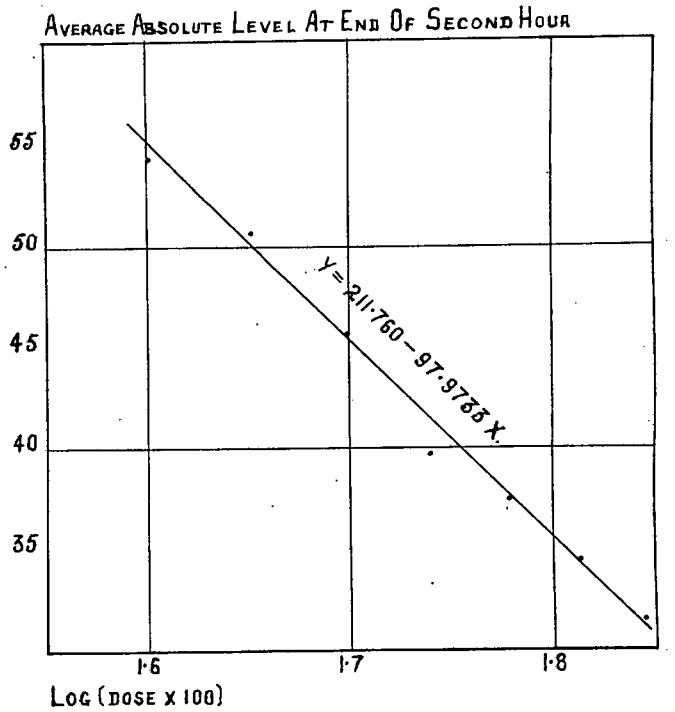


Figure 6. Average absolute blood sugar level at end of 2nd hour.



The discussions in the preceding paragraphs, therefore, furnish evidence that, over the variations of fasting values encountered (0.052 - 0.093 per cent) and in a sufficient number of similarly-treated animals, the initial sugar value can be omitted in the study of the dosage-response curve. However, it is realized that, where the number of test animals is markedly decreased, such a procedure would be open to question, unless the results are compared with those obtained from a corresponding group of animals having received the standard preparation, or with a predetermined curve such as shown in Figure 6. In the method to be described later, both these procedures were used.

Figure 6 represents the dosage-response line (obtained as outlined above) showing the dosage - absolute blood sugar ratio two hours after injection of insulin doses varying from 0.40 - 0.70 unit per 2 kg. rabbit. These limits (i.e., a useful range of 0.40 - 0.70 unit) were arrived at in the following manner: The curve in Figure 3 served to indicate an approximation of the limits of the useful range as from 0.30 - 0. 80 unit. The points, representing the effect to the logarithm of these doses, were determined, whereupon it was found that the best-fitting straight line extended from 0.40 to 0.70 unit only. Inclusion of the lower or higher doses would have resulted in a change of slope of the line and a deviation away from it of those points which now either lie on or very close to it. The straight line further serves to correct those values lying slightly away from it.
On the assumption then that the response is a logarithmic function of the dose, the straight line in Figure 6 represents the true response of rabbits at the end of the second hour to doses of insulin varying from 0.40 - 0.70 units/2 kg.

Bliss and Marks (33) studied the curve relating dosage and graded response. The average percentage blood sugar reduction was plotted against the logarithm of the dose. They subsequently considered the factors whereby the precision of the curve could be increased, and showed that many sources of variation, such as differences between individuals and dates of treatment could be excluded by calculating the results in the analysis of variance, while concomitant variations, such as differences in the initial level or differences in body weight could be corrected more efficiently by the analysis of covariance. By these procedures the following changes were effected in the average percentage blood sugar reduction (quoted from 33):

16.00	corrected	to	17.15
26.55	"	"	26.68
33.04	"	"	32.14
39.02	"	"	38.64.

As can be seen from these figures, corrections brought about by lengthy statistical treatment resulted in comparatively minor changes. In the present series corrections could similarly have been made for variations in fasting values, weights, days of injection, etc, but it was felt that the minor changes to be effected by this would not warrant the time and energy spent for this purpose. Furthermore, in view of the comparatively coarse use the curve was to be put to, such a refinement would have been both unsuitable and superfluous. This curve, previously referred to as the standard insulin curve, was, therefore, without further statistical treatment for correction, adopted as a means of evaluating, by close approximation, the potency of unknown samples of insulin.

/(iii)

(iii) Application of the dose-response curve to the evaluation of potency.

The use of a predetermined dosage-response curve as an aid in the evaluation of the activity of unknown insulin samples, is not a new idea in the field of insulin assay, and has already been referred to somewhat in detail in the discussion of the literature (page 21). Trevan and Boock (17) were the first to make use of such a curve and the application of this procedure was thoroughly investigated by Bliss and Marks (33, 37) and Fieller et al.(38), who pointed out that, notwithstanding slight variations in the slope of the line, it could be utilized, provided each laboratory had, in a series of experiments, determined its own reference curve. However, since the split cross-over, the twin cross-over and the 3-assumption cross-over have come to be almost universally used,and these procedures, by virtue of the variation of doses, each supplies an estimation of its own slope, the use of a reference curve has not assumed practical importance in a detailed accurate assay. It does seem, though, that it could be successfully applied in a quick assay of less accurate nature. The advantage of such a curve is that, for its construction, the effect of graded doses of insulin had been determined on a large number of animals (42 for each dose in this instance), within the limits of probable individual variation, over a long period (18 weeks) under standard experimental conditions (housing, feeding, fasting, treatment, etc). It can be assumed that, if data for a second curve were to be collected under identical conditions, for a similar period, with an equally large number of animals, the slope of the second curve will differ very little from that of the first. The curve actually represents not a single one, as that obtained

/by

by Bliss and Marks (33) from a single assay, but the mean of slope variations that would occur if the data from at least six such assays were pooled. It is fully appreciated that, if for a second and following curves, smaller numbers of animals were to be used, with a shorter dosage range, over a shorter period, the individual slopes of such curves would differ from each other and from that of the standard curve. Consequently, comparison of such slopes or single effects with those of the standard curve will furnish reliable information regarding the value to be attached to an observed effect or an obtained slope.

To evaluate the potency of an unknown sample, the following procedure was followed: The activity of the sample was roughly estimated, using as basis the method of extraction and stage of purification. An amount of insulin, calculated to have an effect falling within the useful range of the reference curve, is injected subcutaneously into 3 rabbits (of the same group as were used to obtain the data for construction of the reference curve, and previously starved for 16-18 hours). Two hours after injection blood is withdrawn and the average absolute level determined. Should this fall well within the useful limits, a provisional value is assigned to the unknown; if not, or if the obtained results from one or more of the rabbits appears inconsistent, the dose is increased or decreased until a satisfactory result is obtained. It was found that with insulin prepared from the same source (e.g., fresh beef pancreas), by standard procedure (e.g., the picric acid-acetonemethod described in Part II), this rough estimation is usually necessary only for the first sample. In subsequent samples the activity generally varies so slightly that this first

/step

step can be omitted. However, should the source be changed or the extraction method modified, it will be necessary to re-introduce the determination of the provisional value. (The picric acid - acetone method was extensively used for the extraction of samples to be assayed by this method. It was found that the yield of crude hydrochloride remained very constant, but that the activity of the hydrochloride varied with the source; thence the suggestion that the rough estimation should be repeated with change of source).

The assay is then continued using six rabbits under the same conditions as mentioned in the preceding paragraph. Three of these receive a dose of insulin standard and three an approximately equivalent dose of unknown (as determined from the provisional value). Regarding the dose of the standard to be used, it is clear that any amount between the limits 0.40 and 0.70 units would serve the purpose. It was felt, however, that the most suitable dose would probably be that which had given the maximum graded response and consequently lie towards the middle of the dose-response line, i.e., 0.50 - 0.60 units. Blood is then collected from each rabbit exactly two hours after injection, and the average absolute blood sugar level determined for each group. The method of activity evaluation is best illustrated by furnishing results of two typical assays:-

TABLE 22.

Assay of beef insulin hydrochloride (crude).

Group A - Dose 0.55 unit insulin standard.			
Rabbit 1	Absolute blood sugar level 2 hours after injection	0.019	percent
" 2	-ditto-	0.049	"
" 3	-ditto-	0.040	"
	Average	0.036	"

/Group B.

TABLE 22 (Continued).

Group B - Dose 0.5 mg. insulin hydrochloride.								
Rabbit 4	absolute blood sugar level 2 hours after injection					0.023	per	cent
Rabbit 5	"	"	"	"	"	0.031	"	"
Rabbit 6	"	"	"	"	"	0.036	"	"
					Average:	0.030	"	"

Calculation:

Log (0.55 x 100) = 1.740

Effect 0.55 unit (from dose-response line) = 0.0413 per cent

Actual effect obtained in assay for standard=0.036 " "

Actual effect obtained in assay for unknown =0.030 " "

∴ Effect of unknown (in terms of reference curve)

$$= \frac{0.030 \times 0.0413}{0.036}$$

= 0.03442 per cent.

Effect of 0.03442 (from dose-response line) is given by dose x 100, the log. of which is 1.81

∴ dose x 100 = antilog. 1.81

∴ dose = 0.65 unit.

∴ Activity of unknown sample = 0.65 x 2

= 1.30 unit/mg.

This value can also be determined arithmetically by using the equation of the straight line. This was done for comparative purposes in several instances, and in each case it was found that the value obtained from the reference curve corresponded so closely with that obtained from the equation of the straight line, that the latter procedure was discarded in favour of the former. If the reference curve is drawn on fairly large scale, this method is as accurate and much quicker.

As assayed by the mouse method, this sample of insulin hydrochloride showed a potency of 1.38 unit/mg.

/TABLE 22(a).

TABLE 22(a).

Assay of pig crystalline insulin.

Group A - Dose 0.60 unit insulin standard.

Rabbit	1	Absolute blood sugar level 2 hours after injection					0.050	per	cent
"	2	"	"	"	"	"	0.049	"	"
"	3	"	"	"	"	"	0.018		
"									
						Average:	0.039	"	"

Group B - Dose 0.028 mg. crystalline insulin.

Rabbit	4	Absolute blood sugar level 2 hours after injection					0.026	per	cent
"	5	"	"	"	"	"	0.034	"	"
"	6	"	"	"	"	"	0.038	"	"
						Average:	0.033	"	"

Calculation:

Log (0.60 x 100) = 1.778

Effect 0.60 unit (from dose-response line) = 0.0375 per cent

Actual effect obtained in assay for standard = 0.0390 " "

" " " " " " unknown = 0.0330 " "

effect of unknown (in terms of reference curve)

$$= \frac{0.0330 \times 0.0375}{0.0390}$$

= 0.0317 per cent.

Effect of 0.0317 (from dose-response line) is given by dose x 100 the log. of which is 1.838

∴ dose x 100 = antilog. 1.838

∴ dose = 0.69 unit.

∴ activity of crystalline insulin = 24.7 units/mg.

Assayed by the mouse method, this sample of crystalline insulin showed a potency of 20.80 units/mg.

Wherever possible, the same animals were used for the assays as those originally used for the standard reference curve. When new ones were introduced, they were subjected to the same preliminary treatment as the older ones had received. With a view to obtaining an approximate even blood sugar in all animals, every care was exercised as regards their housing, feeding, handling, and environmental

/conditions.

conditions. However, even under such circumstances, it is by no means possible to exclude individual variations; they merely serve to, as far as possible, minimize them.

It was realized from the onset that, even for a less accurate assay, the method to be employed must be an indirect one. As Bliss and Marks (37) had expressed it: "Unless it has been shown by prolonged trial that the susceptibility to a specific drug does not vary, the standard should be tested with every sample of unknown under as nearly identical conditions as are practicable experimentally." Such variations to the action of insulin are the common experience of all workers in this field. For this reason both standard and unknown were injected into each of two groups of rabbits. Their action was, therefore, compared under a specific "setting" of conditions which, for the two groups, was as nearly identical as possible. That this "setting" varies from day to day is readily appreciated, and it is to be expected that, on a subsequent day, the amount of response would probably be different. If, for example, on Day X the average absolute blood sugar level of 3 rabbits, two hours after injection of insulin, was found to be 0.034 per cent, it is not uncommon to obtain values of, say, 0.046 or 0.025 per cent on Day Y for 3 other rabbits or even for the same 3. It is largely due to this fact that comparison of an obtained effect with a standard reference curve was introduced. It has already been pointed out that the data for constructing the reference curve were collected from many animals over a long period. The reference curve, therefore, represents not a single but a mean of settings. Observed in a single series, the effect may, therefore, be either above or below the standard effect; by comparison with the reference curve the single effect can readily

/be

be converted to a standard effect. Notwithstanding individual variations, it can be assumed that rabbits receiving insulin at the same time, under identical conditions, will exhibit a nearly identical response. In this method of assay, where only small numbers of animals are used, injection of standard and unknown into two groups serves more for the evaluation of the ratio of response in the two groups (which can be reflected fairly accurately by small numbers) than for the actual amount of reponse (for which more animals are necessary), and which can be obtained more accurately by conversion from the reference curve.

Criticism directed against the method is based chiefly on the following:- (i) the use of too small numbers of experimental animals, and (ii) lack of the cross-over. However, viewed in the light of its general purpose and application, these are not as serious as they appear. The number of rabbits in each group was more or less arbitrarily fixed at three. The consideration of this as a sufficient number is based largely on the observations that (a) on a specific day, under specific conditions, the blood sugar level does not vary appreciably in a small group of similarly-treated animals, and (b) the dose-response ratio is effectively reflected by a small number of animals. Increasing the number of animals in each group would naturally increase the precision and reliability of the method, but this would happen at the expense of extra labour and time involved.

As regards the cross-over, its introduction has been considered the greatest single advance in the assay of insulin. It is fully agreed that no accurate assay can be conducted without it, the percentage error, as shown by Marks (14), being appreciably higher in a direct than in a cross-over experiment. However, even in its

/simplest

simplest form, the cross-over requires at least two days, with the second day preferably spaced one or two days from the first. Its inclusion in this method would merely result in its completely missing the purpose for which it was originally intended. It is felt, though, that the reference curve, to a large extent, compensates for the absence of the cross-over. The specific purpose of the reference curve is to substitute for the cross-over, thus saving time and making the method suitable for a quick assay of an unknown sample.

The final proof of the validity of a method lies in the comparison of its results with those obtained by a standard procedure, or with the standard itself. Of the large number of samples assayed by this method, a relatively smaller number were assayed by the mouse convulsion method as well. Results obtained with the two methods are compared in Table 23.

TABLE 23.

Comparison of assay results.

Sample No.	Mouse method	Rabbit-reference curve method.	Percentage error.
112	1.40	1.50	7.1
6(vi)	0.84	0.92	9.5
138	1.21	1.24	2.4
11	0.48	0.42	12.5
29	0.44	0.48	9.0
12	0.52	0.48	7.6
28	0.30	0.26	13.3
97	2.55	2.08	18.4
44	0.86	0.72	16.3
53	0.78	0.82	5.1
82	1.90	2.06	8.4
84	2.23	2.06	7.6
14	3.36	4.25	26.2
17	3.02	3.84	21.2
148	20.30	23.65	16.5
151	19.04	21.38	12.3
		Average:	11.7

/If

If the results obtained with the mouse method are considered as absolutely correct, then the percentage error of the rabbit - reference curve method calculated from 17 assays is 11.7 per cent, which fully conforms with the requirements of a "coarse adjustment" as originally outlined.

The precision of the method is higher with crude insulin preparations than with the purified material. As a result of the exclusion of the initial blood sugar and a single bleeding after injection, as many as three assays can be conducted per day By one worker and an assistant.

(e) Conclusions.

Small doses of insulin probably show a well-graded effect on the blood sugar. Inability to demonstrate these small changes is the result of (i) technical difficulty and (ii) unsuitable bleeding schedule.

Flattening of the dose-response curve in its lower and upper levels is the result of the time period of testing activity and the bleeding schedule followed. If these were to be adapted to the dosage (i.e., frequent bleedings over a short period for the low doses and less frequent bleedings over a long period for the high doses), the dosage-response curve would show proportionality over a much wider range of doses than is generally accepted.

The response to doses of insulin varying from 0.40 - 0.70 unit can be determined most reliably by post-injection bleeding at 2 hours only, which procedure shows the smallest coefficient of variation. Determination of the blood sugar level at 30 minutes after injection is a measurement of degree in terms of rate of effect, whereas determination at 2 hours post-injection is a measurement of degree in terms of duration of effect. Measurement of the absolute level of the blood sugar after insulin

/administration

administration is, for assay purposes, preferable to measurement of the fall of the blood sugar over a specified period. Initial blood sugar values may be disregarded when determining insulin effect.

Not only the effect (in terms of blood sugar fall) but also the absolute level of the blood sugar after insulin administration is a logarithmic function of the dose. Plotting of the absolute level of the blood sugar against the logarithm of the dose results, for part of the dosage, in a straight line. When the data for this straight line have been obtained from a sufficiently large number of animals, it can be used as a reference curve in evaluating the potency of unknown insulin samples.

The method of assay described is quick, involves less labour and is sufficiently accurate for evaluation of, especially, crude preparations of insulin to within 12 per cent of the correct value.

II. THE PICRIC ACID - ACETONE METHOD OF INSULIN EXTRACTION.

(a) Introduction:

Modern-time conjecture and observations on the pancreas - diabetes mellitus - islands of Langerhans - complex date back as far as 1675. The gradual unravelling of the problem through the fragmentary contributions of a host of workers has been fully described in various monographs on the subject (41, 42, 78) and need not be recapitulated here. It would, therefore, suffice to merely point out in chronological order the outstanding events in the chain of gradual development and unfolding.

1675: Thomas Willis made the observation that diabetic urine was sweet.

1686: Speculating on the function of the pancreas, von Brunner suggested that it was in some way connected with the metabolism of fats and carbohydrates.

1776: Dobson isolated sugar from diabetic urine.

1788: At autopsy of a diabetic, Cowley found sclerosis of the pancreas.

1815: Thomas Bright made observations on the simultaneous occurrence of pathological changes in the pancreas and diabetes mellitus.

1869: Klebs and Munk extirpated the pancreas in dogs with the special purpose of demonstrating a possible relation of the pancreas to diabetes. However, their results were negative.

1869: Langerhans described the islands of the pancreas as epithelial cells distinct from the alveoli and ducts. However, he expressed no opinion on their function.

1875: Peters discovered that acetone is frequently excreted by diabetics.

/1884:

1884: Minkowsky and Kulz found hydroxy-butyric acid in diabetic urine.

1885: von Mering and Minkowski showed that complete removal of the pancreas from normal healthy dogs was followed by a condition closely resembling diabetes mellitus in the human.

1889: Lepine, repeating the work of von Mering and Minkowski, suggested the possibility of an internal secretion of the pancreas as the active agent in the metabolism of fats and carbohydrates.

1892: Minkowski and Hedon established this view when they showed that portions of the pancreas grafted in the abdomen, permitted the gland to be completely removed without serious injury to the carbohydrate metabolism.

1893: Minkowski showed that ligature of the pancreatic ducts, but otherwise leaving the organ intact, caused no hyperglycaemia and diabetes.

1893: Laguesse suggested that the islets of Langerhans were the source of the internal secretion and that injury to the islet tissue was responsible for the origin of diabetes.

1895: Schaffer suggested that human diabetes is caused by a deficiency of the secretion of the islet tissue brought about by some pathological change in the islets.

1901: Gley showed that diabetes resulted when the pancreatic circulation was stopped by tying the veins.

1907: Lane demonstrated A and B cells in the islands of the dog; also that only B cells were destroyed in a diabetic condition.

1909: de Meyer introduced the term "Insulin" to indicate the autocoid of the islet tissue.

Notwithstanding the evidence put forward for an active principle secreted by the islets of Langerhans

/and

and capable of influencing the carbohydrate metabolism, such evidence could not be considered as complete, until the administration of an active extract, prepared from the pancreas, proved effective in alleviating the symptoms in a depancreatized dog or in a diabetic patient. With this object in view many attempts were undertaken which were mostly unsuccessful, due chiefly to the method of extraction or administration. Outstanding events preceding the ultimate successful extraction of insulin may be summarised as follows:-

1892: Minkowski injected pancreatic extracts into depancreatized dogs, but obtained no positive action.

1893: Mackenzie administered the juice from freshly-pressed pancreas orally to three diabetic patients, claiming a general improvement in their health, although the sugar excretion was not affected.

1896: Spillman injected the juice from fresh pancreas and noted a slight improvement in glycosuric condition.

1898: Blumenthal removed protein material from the juice pressed from pancreas by addition of alcohol, but obtained inconsistent results.

1898: Leyden tested pancreatic extract by subcutaneous injection, but the toxicity of his material was too great.

1907: Zuelzer et al. prepared extracts by mincing the glands, adding sodium bicarbonate to produce a weakly alkaline solution and filtering off after a few days. They also extracted minced pancreas from calves with alcohol and obtained promising results on intravenous injection into pancreatectomised dogs and eight patients with diabetes, but the use of these extracts was

/discontinued

discontinued due to untoward symptoms.

1910: Crofton heated the juice pressed from pigs' pancreas for three hours to destroy the enzymes, but with no positive results.

1911: Scott employed strong alcohol as extracting medium so as to prevent the destruction of the active principle by the digestive enzymes. Although the concentration of alcohol he used was such that most of the active principle was not extracted, intravenous injection of his extracts into depancreatized dogs temporarily diminished the sugar excretion.

1913: Murlin and Kramer prepared alkaline extracts of pancreas which lowered the blood sugar of diabetic dogs but it was soon shown that the administration of alkali alone produced a similar effect.

1921: Paulesco prepared an extract of fresh glands with ice-cold water. Intravenous injection brought about a marked improvement in the symptoms observed in depancreatized dogs.

1921-1922: Extraction of the active principle by Banting and Best (1, 4), who clearly demonstrated the ability of these extracts to inhibit glycosuria and hyperglycaemia in depancreatized animals and to alleviate the symptoms of diabetes mellitus in human beings. They first extracted the pancreatic tissue from dogs in which the pancreatic ducts had been ligated for ten weeks, thus causing degeneration of the acinar tissue, with Ringer's solution. Following this, they macerated the pancreae of foetal calves with Ringer's solution and afterwards with alcohol, thus again obtaining potent extracts. They were guided in this respect by the earlier observations of Carlson and Drennan (91) to the effect that, up to about the fourth month, the foetal calf pancreas contains no proteolytic enzymes. Finally, in collaboration with

/ Collip.

Collip they obtained active extracts by using acidified alcohol as extracting medium, thus preventing enzymatic action. The subsequent fractional precipitation devised by Collip yielded more potent and less toxic extracts.

(b) Extraction Methods:

The existence of the pancreatic hormone had been proved and Banting and co-workers had afforded a general method of preparing active extracts. During the years 1923 and 1924 a flood of new methods of preparing and purifying pancreatic extracts poured into the literature and a steady progress along these lines has since been maintained. Most of these procedures were based on the extraction of freshly minced pancreas with acidulated or alkaline solutions: aqueous, acetone, methyl alcohol or ethyl alcohol. Of these, only alcohol has assumed practical importance as extracting medium, so that at present it is almost universally used for this purpose. Aqueous extraction was attempted by Paulesco (116) and afterwards by Best and Scott (43), whose results, although encouraging, could not show the same unitage per pound of pancreas as did the alcohol methods. Murlin and co-workers (44) used 0.2N aqueous hydrochloric acid as extracting medium, Dodds and Dickens (45) a 1 per cent aqueous solution of formic acid, and Kaulbersz (46) a 0.02N aqueous sodium bicarbonate solution, but in all these the yields were well exceeded by those of alcoholic extractions. Moreover, in these methods the filtration from the minced glands is usually very slow, so that the proteolytic enzymes destroy a large portion of the active principle.

While most workers have retained alcohol as extracting medium, many variations were introduced so as to

/increase

increase the ultimate yield. These were concerned especially with the nature and strength of the acid used, and with the means of precipitating the active principle. Collip (47) (48) had shown that insulin is soluble in 80 per cent alcohol but insoluble in alcohol of more than 90 per cent. He consequently extracted pancreas with an equal volume of 95 per cent alcohol and precipitated the inactive protein from the filtrate by the addition of two volumes of 95 per cent alcohol. Moloney and Findlay (49, 50) extracted with an alcoholic solution of benzoic acid, precipitated the active fraction by the addition of sodium benzoate and subsequent acidification with hydrochloric acid. Best and Scott (51, 52) extracted with alcohol acidified with 1.3 per cent acetic acid and precipitated the insulin with ether. Shonle and Waldo (56) used trichloracetic acid as precipitating agent, while others employed potassium lactate and potassium ferrocyanide (117) for this purpose. Somogyi, Doisy and Schaffer (53) used strong acid during the alcoholic extraction and precipitated the hormone by half-saturation of an aqueous solution with ammonium sulphate. Blatherwick et al. (54) and the Toronto workers (55) substituted sodium chloride for ammonium sulphate in precipitating the insulin. The latter also confirmed the opinion that alcoholic hydrochloric acid is the best extractant for insulin. Jephcott (57) in investigating the method of the Toronto workers, found the optimal concentration to be from 60 - 80 per cent for alcohol and from 0.12 - 0.33N for hydrochloric acid, with somewhat higher values for sulphuric acid. Excessive acidity resulted in serious loss of potent material.

Following closely on the heels of the improved methods of extraction were the methods of purification of the crude material. These consisted essentially of one or more of the following procedures:

/(1)

(i) fractional precipitation with organic solvents; (ii) precipitation as a salt; (iii) iso-electric precipitation; (iv) adsorption.

Somogyi et al. (53) and Waldo (56) independently observed that most of the active principle can be precipitated from aqueous solutions by adjusting the hydrogen ion concentration to about pH5. The degree of purification by this method is fundamentally dependant on the iso-electric points of the constituent protein materials. This has become one of the standard methods of purification of insulin, although the potency cannot be increased beyond a certain stage by repeated iso-electfic precipitations. Somogyi and co-workers (53) found their purest sample to have an iso-electric range of pH4.4-5.8 with a maximum at pH5.0. Shonle and Waldo (56) found pH4.3 to 5.7 and Blatherwick et al. (54) a higher range of pH5.8-6.0. According to Abel (58), the iso-electric point of crystalline insulin is pH5.5-5.6. Howitt and Prideaux (59), using the method of electrophoresis, found that the zone of insolubility extended from pH4.5-6.5 and the iso-electric point was at pH5.4.

The method of extraction and purification as used by the Toronto workers has been described by Scott and Parker (55). They extracted with 60-80 per cent alcohol containing 1 per cent hydrochloric acid and subsequently salted out the active material by the addition of sodium chloride first to a concentration of 25 per cent and then (after redissolution) to a concentration of 15 per cent. This is followed first by iso-electric p recipitation, then by fractional precipitation with alcohol and ether and, finally, again by iso-electric precipitation. With this procedure a yield of 2,000 units per kilo of ox pancreas at an activity of 16-17 units per milligram is claimed.

/Gerlough

Gerlough and Bates (60) obtained a product assaying at 21-24 units per milligram by extracing with alcoholic hydrochloric acid and salting out of the insulin protein at pH2.5-2.8 by 16 per cent anhydrous sodium sulphate. This is followed by fractional precipitation with alcohol first at 60 per cent and then at 90-92 per cent and, finally, by double iso-electric precipitation.

Dudley (61) purified crude insulin, prepared by Collip's method, by precipitating the active material from aqueous solution with picric acid and subsequently converting the insoluble picrate to the soluble hydrochloride. Dickens et al. (62) precipitated the active material with 2 per cent trichloracetic acid, salted out the insulin from the acid solution and precipitated it as an oxalate at pH2.4-3.7 by the addition of N/8 potassium oxalate, in this way obtaining a material for which they claimed a potency of as much as 30 units per milligram. Dingemanse and Laqueur (63) purified insulin by adsorption on charcoal and replacement by phenol, the complete process consisting of electrodialysis, fractional solvation with sodium bicarbonate, adsorption and fractional elution with disolium phosphate. They claimed to have obtained a material with a convulsive dose of 0.003-0.0045 milligram.. Abel and Geiling (64) purified insulin by repeated precipitation with N/6 pyridine solution from a dilute acetic acid solution. The dry precipitate was extracted with 90 per cent phenol and the insulin precipitated from solution by the addition of dry ether or absolute alcohol. In the method of Kharasch (65) the dry insulin powder is treated with anhydrous liquid ammonia and the mixture filtered. The less active material is insoluble, while the more active fraction is recovered by evaporation of the filtrate.

/A

A recent method (1949) for the extraction and purification of insulin has been advanced by Lautenschalager and Linder (66). This consists of extraction with alcohol and hydrochloric acid at 0-15°C. The extract is adjusted to pH7.5 with ammonia, the precipitate separated by centrifugation, the pH adjusted to 3 and the liquor concentrated by evaporation in vacuo. The active principle is then twice salted out with sodium chloride, redissolved and the insulin isolated at pH6-8.

The crowning achievement in the purification of insulin seems to have been reached when Abel (67) isolated the hormone in crystalline form. This was obtained by dissolving purified insulin in weak acetic acid and removing impurities by precipitation with brucine. On addition of N/6 pyridine crystalline and amorphous insulin separated together. In a later communication Abel and his co-workers (68) claimed better results with a slightly modified technique. N/6 pyridine was added to the solution as above, but the precipitate was removed by centrifugation and the clear fluid treated with ammonia; the precipitate was filtered and the filtrate, on standing, deposited crystals of the active principle. In subsequent publications (68-70) these workers prepared crystalline insulin without the use of brucine, thus obtaining a material assaying at 24 units per milligram (71). Harington and Scott (72) devised a further method of obtaining crystalline insulin by substituting saponin or digitonin for brucine and precipitating with ammonia in much the same way as Abel and his co-workers. Their material as assayed by Culhane, Marks, Scott and Trevan (73) consistently showed an activity of 23-27 units per milligram. Scott (74) proved the dimorphous nature of the crystals having obtained a wedge-shaped form on crystallisation from

/acetic

acetic acid-ammonium acetate buffer in aqueous acetone at pH5.2 and rhombohedra with the same procedure at pH6.2. In 1934 Scott (75) discovered that crystalline insulin, prepared by the above methods, contained zinc and that the addition of small quantities of other metals (salts of cadmium, nickel, cobalt) facilitated the crystallisation. Scott and Fisher (76) determined the nature of the ash of samples of insulin crystallised from media containing zinc, cobalt and cadmium salts, obtaining constant values for the respective metals. Scott pointed out that the previous successes in crystallising insulin were due to the fortuitous presence of one of these essential metals. Romans, Fischer and Scott (77) subsequently published the outline of a procedure which starts with the fresh pancreas and gives crystalline insulin as the final product. This method yields 800-900 international units of crystalline insulin per pound of beef pancreas.

Studies on the chemistry of insulin followed closely on the heels of the improved methods of extraction and purification.

Notwithstanding claims to the contrary, insulin was first regarded as a protein by, inter alia, Best and Macleod (95). It was definitely placed in the protein and metaprotein group by the work of Dickens, Dodds, Lawson and Maclagan (96). Svedberg and Sjögren (97) working on the molecular weight of insulin, concluded that it is a homogeneous protein of the ovalbumin type. On the basis of hydrolysis of crystalline insulin with mineral acids, Jensen and Wintersteiner (98) gave the tentative distribution of amino acids as follows, thus accounting for 88 per cent of the molecule:-

Leucine 30%, glutamic acid 21%, tyrosine 12%, cystine 12%. histidine 8%, arginine 3% and lysine 2%. Recently the

/following

following complete analyses of the amino acid content of insulin have been reported by Chibnall (99) and Brand (100). (Figures in per cent of ash and moisture-free protein) :-

Constituent	Chibnall	Brand.
Arginine	3.00	3.50
Histidine	4.88	5.30
Lysine	2.44	2.60
Glutamic Acid	18.60	20.20
Aspartic Acid	5.68	6.80
Amide N (NH_2)	1.68	2.15
Cystine /2	12.50	11.00
Tyrosine	13.03	12.30
Alanine	4.60	2.90
Valine	7.49	8.80
Phenylalanine	8.09	7.90
Serine	5.18	5.80
Threonine	2.08	3.20
Leucines	15.68	16.30
Glycine	4.30	4.60
Proline	2.56	2.90
Cysteine		0.60.

The data by Velick and Ronzoni (101), presenting a complete analysis for the amino acid composition of insulin, are in good agreement with those cited above.

Abel et al. (58) considered the elementary composition of insulin similar to that of the average protein. At an early date the presence of nitrogen was established and values ranging between 13.7 and 18.43 per cent were recorded. Abel (58) found a nitrogen content of 15.40 per cent for crystalline insulin, while that found by Brand (100) is 16.04 per cent. Dudley (80) first suggested the presence of sulphur in the insulin

/molecule

molecule. The total sulphur content of crystalline insulin has been estimated at 3.15 per cent by Abel (58), 3.27 per cent by Jensen et al. (71) and 3.11 per cent by Harington and Scott (102). The value as obtained by Brand (100) is 3.31 per cent. It has been established that all the sulphur of insulin is present as a disulphide linkage and can be accounted for as cystine (103). Reduction of the disulphide linkages in the insulin molecule under various experimental conditions, results in a loss of physiological activity (41, 42, 104). The elementary analysis, as given by Brand (100) is: Carbon 52.96 per cent, hydrogen 6.79 per cent, nitrogen 16.04 per cent and sulphur 3.31 per cent.

The empirical formula derived by Abel (58) for his crystalline insulin is $C_{45}H_{75}O_{17}N_{13}$. Svedberg and Sjogren (97,105), using the ultracentrifuge method, investigated the molecular weight of insulin and found that it changes with the pH value of the solution. Within the range pH4.5-7.0 the insulin is stable, but outside it, it dissociates into products of lower molecular weight, this dissociation being reversible if it has not been kept too long outside the range. At pH6.7-6.8 the mean value of the molecular weight was 35,100. Crowfoot (106) obtained a value of 37,200. The molecular weight estimated from data obtained on redetermination of ultracentrifugal sedimentation and diffusion constants of carefully recrystallized insulin was found by Miller and Andersson (107,108) to be 46,000. Gutfreund (109) confirmed his previous observations that insulin in solutions of pH7-7.5 was homogeneous from the point of view of particle size and had a maximum molecular weight of 47,000-48,000. At pH above 7.5 or below 4 evidence was obtained for reversible dissociation of the hormone,

/the

the optimum pH for dissociation being between 2 and 3. The minimum molecular weight from osmotic pressure determinations was 12,000 ± 500. He concluded that insulin consists of subunits with a molecular weight of 12,000 and that the forces holding these subunits together involve the ionizing groups of the protein.

Sanger (110) found that an insulin submolecule of molecular weight of 12,000 contained two glycine and two phenylalanine residues containing free α-amino groups, and two lysine residues containing free ϵ-amino groups. These results suggest that the insulin submolecule is made up of four open polypeptide chains, two of these having terminal glycyl residues and the other two terminal phenylalanine residues, the chains being bound together most probably by —S—S— linkages.

The same author (111) applying the performic acid oxidation method to the ox, pig and sheep insulin submolecule, obtained findings suggesting that, contrary to general opinion, chemical differences do exist among highly purified insulin preparations of different species. Amino acid analyses of the acidic fractions revealed considerable differences in the contents of serine, glycine, theeonine and alanine. Threonine was found to be present in the acidic fraction of pig insulin but absent from the same fraction of ox and sheep insulins.

As regards the question whether the protein is identical with the hormone itself or acts simply as a convenient carrier on whose surface the true hormone is adsorbed or to which it is loosely bound, conclusive evidence is lacking. Attempts have been made to interpret the results obtained on treatment of insulin with various reagents, as indicating that certain groups of the insulin molecule, such as phenolic hydroxyl, primary amino and the disulphide linkage are essential for the physiological activity of the hormone. "The pharma-

/codynamic

codynamic function of insulin may be due to

(i) the presence of a prosthetic group in the molecule, but no evidence for such a group has as yet been obtained;

(ii) the occurrence in the insulin molecule of an unknown specific amino acid, but thus far only known amino acids have been isolated from insulin;

(iii) the existence in the protein molecule of a specific grouping of certain component amino acids embedded in the molecule which, by virtue of their chemical and spatial configuration, impart a specific pharmacodynamic function to the protein molecule.

For this reason the entire molecule is necessary for the physiological activity of the protein" (112).

The bulk of evidence available favours the latter opinion, that the hypoglycaemic activity of insulin is a specific property of the whole protein molecule, and any reaction which may produce a change in the architecture of the protein molecule is likely to cause a loss of physiological activity. The reactions of insulin with iodine (113) and reducing agents (114) indicate that tyrosine and disulphide groups are necessary for the hormone's hypoglycaemic action. The work of Reitz et al. (115), who have prepared active sulphate esters of insulin by treatment with concentrated sulphuric acid, shows that either the aliphatic hydroxyl groups or the net charge do not participate in the biological action of insulin.

The physical properties of insulin, further chemical properties, its inactivation and reactivation and the action of various reagents and enzymes on it have been dealt with at length by various authors (41, 42, 112).

Crystalline insulin represents the pure iso-electric product. Addition of either acid or alkali to an iso-electric precipitate of insulin results in complete

/solution,

solution, owing to the formation of the acid or basic salt, according to the generally accepted theory of amphoteric proteins. The most commonly known salts of insulin are the hydrochloride, the picrate and the sulphate. Of these the sulphate only has been prepared in an inactive crystalline form.

Regarding the effect of insulin when injected into animals, this has been referred to in a previous section (see page 3). The endocrine function of the pancreas and the physiological action of insulin have been reviewed in detail by various authors (41, 42, 112, 127, 130, 136).

Since it falls outside the scope of this investigation, not more than casual reference can be made to it. The total sum of the chemical changes which occur in the tissues of the body, i.e, inorganic and organic metabolism, is controlled by the proper physiological co-ordination of factors such as hormones, vitamins and enzymes. "As regards the role of hormones, they apparently do not initiate new metabolic processes, but rather influence the rate of speed of existing processes by accelerating or inhibiting certain enzymatic reactions in the cell upon which they act" (112). That insulin is intimately linked up wth the organic metabolism is especially evident from the physiological disturbances in the body which are observed in total pancreatectomy or in diabetes mellitus:-

(i) Pronounced hyperglycaemia and glycosuria;

(ii) Depletion of glycogen stores in certain tissues (liver, muscle);

(iii) Lowering of the respiratory quotient, indicating a decrease in the rate of the oxidation of glucose;

(iv) Increase in the NPN of the urine, which is due to an increase in the conversion of protein into glucose;

(v) Increased formation of ketone bodies, caused by an acceleration of fat catabolism.

/Insulin

Insulin acts on (i) the liver by inhibiting glycogenolysis and gluconeogenesis and by stimulating the conversion of glucose to fat and (ii) on the liver, muscle and other tissues by promoting the storage of glycogen and stimulating, indirectly, the oxidation of glucose. It, therefore, stimulates those processes causing glucose to enter the blood with the over-all effect of a lowering of the blood sugar. In this respect it is antagonised by the hormones from the pituitary, adrenal and thyroid (137). Certain adrenal and pituitary hormones tend to increase carbohydrate formation from protein by the liver and to diminish the peripheral utilization of glucose (138-142). These hormones (insulin, pituitary, adrenal) exert their effect on the peripheral utilization of glucose in the following way:-

The effects of insulin on the utilization of glucose are demonstrable in many types of intact muscle (eviscerated animals, isolated perfused heart, isolated rat diaphragm) and even in cell-free extracts of liver, brain and heart tissue,(143-151), the fate of the glucose depending on the particular preparation used: it may undergo complete oxidation or it may be converted largely to glycogen. These are the two fundamental effects of insulin on peripheral glucose utilization.

The proximate fate of glucose disappearing in the tissues is conversion to glucose-6-phosphate. This phosphorylation of glucose has been extensively studied, particularly by the Coris and their co-workers (143). This is probably due to increased activity of the enzyme hexokinase and there is no doubt that the main reaction consists in an exchange of phosphate between adenosine-triphosphate (ATP) and glucose, through which glucose is converted into glucose-6-phosphate. which may be either

oxidized into pyruvic acid or polymerised into glycogen, and ATP into adenosine diphosphate or into adenylic acid. "The main action of insulin, therefore, appears to be an activation of a complex enzymatic system which fixates blood glucopyranose and converts it to a labile, less dextro-rotatory form, possibly fructo-furanose; it borrows the energy necessary to this conversion from the oxidation of various carbohydrate derivatives, particularly of pyruvic acid. The activated glucose subsequently reacts with adenosine-triphosphate and is transformed into glucose-6-phosphate" (127).

Price, Cori and Colowick (152) found that the rate of hexokinase reaction could be depressed by the addition of certain protein fractions obtained from the pituitary. This inhibition was much more marked when the pituitary fraction was supplemented with adrenal cortex extract (153). The inhibition is dependent upon the presence of a very labile protein, presumably of pituitary origin, since addition of suitable pituitary extracts results in inhibition of phosphorylation either in the presence or absence of added adrenal cortex extract. Addition of insulin to either of these inhibited systems re-establishes normal phosphorylation; potassium hydroxide treatment of the insulin abolishes its effectiveness. The labile pituitary factor is not identical with either purified adrenotropic, lactogenic or growth hormones. Broh-Khan and Mirsky (154) obtained similar, though more fluctuating, results; they also found that a substance causing hexokinase inhibition can be extracted from the spleen.

It appears, therefore, that insulin is not essential for glucose utilization but serves rather to oppose the inhibitory action of anterior pituitary and adrenal cortex hormones on this process. It seems that some of the effects

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of hormones on carbohydrate metabolism in the liver cannot be explained in terms of regulatory effects on the hexokinase reaction, and it is likely that other enzymes such as liver phosphorylase, stimulating glycogen formation from glucose-1-phosphate, will be found to be similarly under endocrine control.

The use of picric acid in connection with insulin was first described in 1923 by Dudley (61), who used it for the purification of crude insulin prepared by a modification of Collip's method. The crude material obtained by this method was found by Dudley to have an activity of 5-10 milligrams = 1 Original Toronto Unit. In the purification process a 1.5 per cent aqueous solution of this crude insulin is made. To this is then added half its volume of a saturated aqueous solution of picric acid, resulting in an immediate flocculent amorphous precipitate, the insoluble insulin picrate, the yield being about 7.5 per cent of the crude insulin taken. The picrate is then transformed into insulin hydrochloride by grinding it up in a mortar with absolute alcohol containing 5 per cent (by weight) of hydrochloric acid. To the clear filtrate is added dry ether and the hydrochloride settles as a white amorphous powder, the yield obtained being 75-80 per cent of the picrate, and the potency of the material 0.5-0.75 milligrams = Toronto Unit (rabbit unit). In the same paper (61) Dudley states that if iso-electric precipitation is carried out with a 2 per cent solution of hydrochloride a precipitate forms at pH5.7, carrying with it about 50 per cent of the activity and having a potency of 0.3 milligrams = 1 rabbit unit. In a subsequent publication Dudley and Starling (80) described an improved

/technique

technique for the conversion of picrate to hydrochloride, which consisted of dissolving the picrate in a solution of hydrochloric acid in 75 per cent alcohol (25 mls. 3N (aqueous) hydrochloric acid + 75 mls.absolute alcohol) and precipitating the hydrochloride by the addition of 10-20 volumes of dry acetone, the rabbit unit proving to be as low as 0.2 mg.

In 1924 Dudley (79) used picric acid for the extraction of insulin from the islet tissue of the cod. The results of McCormick and Noble (81) indicated that the amount of insulin in pancreatic tissue preserved in 70 per cent alcohol containing 0.3 per cent hydrochloric acid, diminished according to the time elapsing before the material was worked up. Similar observations were made by Dudley. In considering alternative methods of collecting the material, he made use of a saturated aqueous solution of picric acid and obtained remarkable success. The fish islet tissue was preserved in this fluid for periods varying from 6 to 12 days, after which it was ground in a mortar with 75 per cent acetone. This was evaporated in vacuo and the precipitated picrate converted to the hydrochloride, having an activity of 1 milligram = 1 rabbit unit and a yield of 13.12 rabbit units per gram of wet tissue (13120 rabbit units per kilo). Dudley states that "it is possible that this method will prove applicable to the commercial production of insulin from slaughter-house material. It appears that once the fresh islet tissue has been dropped into an adequate quantity of this fixative, it can be kept for any time determined by convenience, transmitted without refrigeration to the factory where the extraction of the insulin is to be carried out, and still give a yield at least as good as that obtained with the greatest precautions as to speed and temperature, when the alcohol process of preservation is used."

Coincident with this work Dodds and Dickens (82), working on a water extraction method, were investigating the possible use of picric acid in this respect. They extracted freshly-collected pancreas with 1 per cent aqueous formic acid and precipitated the dissolved proteins by addition of an equal volume of saturated aqueous picric acid. The picrates were subsequently extracted with 70 per cent acetone, insulin picrate dissolving to the partial exclusion of other protein picrates. This was reprecipitated by dilution with equal volumes of saturated aqueous picric acid and water, and converted to the hydrochloride which showed an activity of 1.5-2 mg.= 1 rabbit unit and gave a yield of approximately 300 rabbit units per kilogram of pancreas. Endeavouring to inhibit tryptic action, the authors subsequently added 5 per cent paraldihyde to the extracting fluid, obtained a hydrochloride with an activity of 2 mg = 1 rabbit unit and a yield of 535 rabbit units per kilo of material. However, it became evident that about 40 per cent of the yield was lost through incomplete precipitation of the picrate by dilution. Following the procedure as outlined above, but evaporating the acetone by distillation in vacuo, instead of dilution, the yield was increased to 1,040 rabbit units per kilo of pancreas. Having been informed by Dudley at this stage of the method under trial by him for the extraction of fish insulin, they omitted the preliminary aqueous extraction and applied the picric acid directly to the original tissue. The general outline of the method was to mince the pancreas with the addition of dry, powdered picric acid during the mincing process. After thorough mixing the mixture was extracted with 70 per cent acetone and the latter evaporated by distillation in vacuo. The precipitate of picrate having been washed with ether to remove fat, was converted into the hydroc' The activity of the hydrochloride so obtained varied

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1-0.25 mg = 1 rabbit unit and the yield from 1,000-1,845 rabbit units per kilo of pancreas (pig).

The important underlying principle in this method is the specific solubility claimed for insulin picrate in aqueous acetone. This was first commented on by Dudley and Starling (60) and subsequently extensively studied by Dudley (79) and Dickens and Dodds (83). The optimum concentration of acetone for the solubility of insulin picrate was found to be about 70 per cent. Above and below this concentration the solubility decreases markedly, "which indicates that insulin picrate possesses very sharply-defined solubility in mixtures of acetone and water" (83). Dickens and Dodds prepared a series of picrates from blood, egg-albumin and pancreatic proteins. "Experiments on the solubility showed that the majority of protein picrates are insoluble in either absolute or aqueous acetone, insoluble in absolute alcohol and sparingly soluble in aqueous alcohol. The solubility of insulin picrate is, therefore, very different from that of protein picrates; thus it was found that insulin could be separated from associated proteins by extraction of the mixture of picrates by means of aqueous acetone" (83).

The final method of extraction adopted by Dickens and Dodds (83) is the following:-

"The pancreas is minced and well stirred with finely-powdered picric acid (45 gm. per kilo of pancreas, which has been previously drained on a Buchner funnel). The mixture is again passed through the mincer to ensure even mixing, and it may be advantageous, particularly with frozen pancreas, to mince once more.

"From the well-mixed picrated mass the picrate is then extracted with acetone. Three extractions are necessary and the concentration of acetone in the extracting

/fluid

fluid should be 70 per cent approximately. The quantity of acetone used in the first extract will vary with the amount of water present in the picric acid - pancreas mixture.

"The minced pancreas-picric acid mixture is stirred with the requisite quantity of acetone and efficient mixing is ensured by passing the whole mass through the mincer once again. This mixing should produce a paste of creamy consistency, from which the liquid extract is pressed in a suitable press through a double layer of "jean". When no more liquid can be pressed out, the solid material remaining is again minced into the required volume (equal to half the weight of pancreas taken) of 70 per cent acetone and re-extracted as before. This process is repeated once more.

"The combined extracts are filtered, if necessary, and the filtrate distilled in vacuo until all the acetone is removed. From the aqueous residue, after cooling, a deposit of the amorphous precipitate of the picrate together with some fat and crystals of picric acid separate. The precipitate is collected on a Buchner funnel, washed by stirring with ether and the ether filtered off. The picrate remains undissolved whilst the fat and excess of picric acid are removed by washing with ether. So obtained, the picrate is a p ale yellow, amorphous powder which is readily converted into the hydrochloride.

"For this purpose it is dissolved in acid alcohol prepared by mixing 25 cc. of aqueous 2NHCl with 75 cc. of alcohol. Ten to twenty cc. of this mixture are usually required for each gramme of picrate. By careful rubbing with a glass rod, a turbid solution of a dark brown colour is obtained. The solution is then centrifuged and the supernatant fluid is poured off. The solid material in the bottom of the tube is ground up with a further

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quantity of the acid alcohol solution, and is centrifuged again. From the resulting clear fluid the hydrochloride is precipitated by the addition of ten to twenty volumes of acetone. The hydrochloride is allowed to settle and the clear supernatant fluid is decanted. The remainder is poured onto a Buchner funnel and the precipitate is washed with acetone until free from picric acid, and, finally, with dry ether. It is then dried in a vacuum desiccator. The crude hydrochloride so obtained is a perfectly white, non-hygroscopic, amorphous powder, the rabbit unit of which lies between 0.25 to 1 mg."

An additional feature of the method is the apparent insensitivity of the picrate to higher temperatures. With distillation in vacuo at 40°C the authors obtained 675 rabbit units per kilo from a particular sample. With distillation at ordinary pressure from a water-bath at 80°C, they obtained 712 units per kilo. The time to complete the process is about one day, the authors having worked up batches of up to 10 kilos within six hours.

A similar method has been worked out independently by Bordelli and Deulofeu (84, 85) and Wernicke (86).

These are the last communications with a direct bearing on the picric acid-acetone method of insulin extraction. In monographs on the subject (41, 42, 78) this method is cited as a standard method of insulin preparation; other workers occasionally refer to it in passing by as an alternative method of insulin extraction (87), but since the work and claims of Dudley, Dickens and Dodds, very little direct evidence has been forthcoming to either substantiate or disprove these claims.

The acid alcohol method with minor or major modifications appears to be almost universally used for both the experimental and commercial production of insulin.

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During the early phases of experimental work on insulin production, many and varied methods were proposed. One by one these were discarded in favour of the acid alcohol method, the reasons for this being, in most cases, quite obvious, these including, inter alia, too low a yield, enzymatic destruction of the active principle during the process of extraction, long and tedious filtrations making the method impracticable and cumbersome, impossibility of repeating the experimental method on large scale, high production costs or some other distinct disadvantage as compared to the acid alcohol method. None of these have authoratitively been forthcoming for the picric acid-acetone method and yet it seems to have almost completely fallen into disuse except in isolated cases.

The advantages claimed for this method are such that they cannot be overlooked without due consideration. Foremost of these are the fixative qualities of picric acid and the claimed stability of the picrate. In the acid alcohol method the first essential is the immediate working up of the freshly obtained pancreatic material, or, alternatively, preservation of material by freezing, as the alcohol cannot serve this purpose due to its untoward effects on the insulin content (79, 81, 88). With commercial concerns this necessitates the speedy removal of the material from the abattoir to the factory or, alternatively, the freezing of the material at 20-30°C and storage thereof until such time that it can be transported to the extraction plant. This, in its turn, requires the presence of freezing facilities at those depots from which pancreas is collected for extraction purposes. While this may not be a serious obstacle as far as the larger centres are concerned, it does

/certainly

certainly assume practical importance when the attention is focussed on the smaller centres where the total obtainable material is insufficient to warrant the establishment of costly refrigeration plants. In the face of a low world production of insulin and an increasing world consumption of the product as illustrated later in this work, it appears that at some future stage insulin-producing concerns will be compelled to utilize all available material. This defect (lack of refrigeration facilities) is especially acute in thinly-populated areas and countries which lack large and well-equipped slaughter houses. Convincing evidence for this statement and the importance of the above argument is obtained when considering the production figures quoted for Germany after introduction of the anhydrous sodium sulphate method of preservation (89). This process, developed in the laboratories of the Farbwerke Hochst by Lindner is based on the principle of converting the pancreas glands into a stable dry product by binding their water content as water of crystallization with anhydrous sodium sulphate. The latter is added to the pancreas during the mincing (700 gm. anhydrous sodium sulphate per kilo of material) and the minced mass packed on iron sheets in a layer of about 5 centimetres thick. It is placed in a refrigeration chamber overnight and may thereafter be stored for as much as 6 months at 5-8°C or for several days at room temperature not exceeding 30°C without loss of insulin. By applying this process the Farbwerke Hochst were able to double the number of slaughter houses from which they obtained the necessary pancreas for the production of insulin. This method has done much to stave off the worst consequences of the insulin shortage for diabetics in Germany.

"It seems clear that chemical dehydration will enable insulin to be extracted from pancreas which would otherwise be wasted and that it should help to increase the supplies of insulin in all countries in which the abattoirs have no means of freezing the fresh pancreas" (118).

Further evidence is supplied by the following: During the war years a large consignment of frozen pancreas was exported from Rhodesia to Great Britain. The Rhodesian Railways were, at the time, unable to supply refrigeration trucks and, although the material was packed in ice for transport and shipped under refrigeration, the entire consignment was a total loss (90). Added to this the higher cost of transport and shipment under refrigeration must also receive consideration.

It is, therefore, apparent that any method claiming to obviate initial freezing of the material must be taken seriously into account. Although the anhydrous sodium sulphate method (loc. cit.) partly fulfils this requirement, it is cumbersome and unfit for use for periods longer than several days without refrigeration.

A further aspect of the usefulness of such a method is furnished by the following:

In order to determine, for research purposes, the insulin content from a particular source of pancreas, it is sometimes unavoidable that the material be collected outside the reach of freezing facilities. This was experienced when, in the present work, the pancreas from a particular type of shark was investigated for its insulin content. The material was collected on the trawlers for periods varying from one to six days. These, like most fishing boats, were not supplied with refrigeration installation and, in view of this, the

/project

project had either to be abandoned or the picric acid-acetone method reverted to. Moreover, it is not impossible that at some future stage insulin will be produced for commercial purposes from fish pancreas. This will necessitate either supplying all trawlers with freezing apparatus, as is done at present in Norway (87), or, alternatively, finding a method not requiring preliminary freezing.

Further advantages claimed for this method include its ease and rapidity of working, the higher yields obtained as compared to the acid alcohol method and the possibility of evaporating the acetone at normal pressure and high temperature instead of the conventional in vacuo and temperature at low point.

It is these considerations that prompted the present critical investigation of the picric acid-acetone method.

(c) Method.

The different procedures in this method of preparation that were fully investigated are the following:-

1. Time factor in fixation;
2. Effect of temperature on picrated mass;
3. Effect of acetone on picrate;
4. Optimum concentration of acetone;
5. Pressure and temperature during process of distillation;
6. Conversion of picrate to hydrochloride;
7. Stability of hydrochloride;
8. Purification of hydrochloride.

Pancreatic material used for this investigation was obtained from the local abattoir, consisted chiefly of beeves and, to a lesser extent, of sheep and pig pancreas. Having been simultaneously engaged on an investigation of

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the insulin content of the whale pancreas, some of the results quoted hereafter refer to the latter. However, the source of pancreas used is clearly indicated in each case.

Pancreases were removed from the animals as soon as they were killed and the material rushed to the laboratory for mincing. Prior to this they were carefully trimmed by removing excess connective tissue, fat, glands and liver strips. The lapse of time from the death of the animals until the mincing of the material in no case exceeded 1½ hours. During the mincing the crystals of picric acid (which had previously been drained on a Buchner funnel) were added to the material (the amount varying from 60 to 110 gm. wet weight per kilogram of material), and the minced material constantly stirred. Following the first mincing the entire mass was re-minced a second and a third time. The water which separated during the process was in no case discarded for fear of losing active material.

The minced, watery, picrated mass was constantly stirred to ensure even distribution of the water. During the stirring samples of exactly 200 gm. were weighed out and placed in small jars, as many samples being prepared as required for the particular purpose. In each batch of samples the contents from one specimen jar were used to determine the water content of the batch. For this the jar plus contents were weighed and the open jar placed in an incubating oven at 65°C for 2 to 3 days until quite dry and then weighed again. On these results were based the calculations for the acetone concentration used in each instance. In those cases where the extraction was commenced before the determination of the water content, the latter was estimated and corrections made after it had been determined.

The method of extraction closely followed that of

/Dickens

Dickens
and Dodds, the details being as follows:-

The water content of a particular batch having been determined, the volume of acetone added for the first extraction was calculated in order to give the required concentration of acetone. To illustrate a typical example:

Water content of sample: 140 c.c. per 200 gm.

Desired concentration: 60 per cent acetone.

$\therefore$ add to sample $\frac{140 \times 60}{40} = 210$ c.c. absolute acetone (i.e., for first extraction).

After the required period for the first extraction the picrated acetone mixture was transferred to a hand press and as much fluid as possible pressed out. To the dry residue was added 200 c.c. of 60 per cent acetone for the second extraction and the process repeated. After this followed a third extraction with a further 200 c.c. of 60% acetone. The combined turbid press liquids (measuring about 650 c.c.) deposited a fine insoluble precipitate on standing. The clear supernatant fluid was decanted and the remainder filtered through a Buchner funnel.

The clear filtrate of aqueous acetone was transferred to a distilling flask and the acetone evaporated at reduced pressure and temperature not exceeding 30°C (unless otherwise stated in the results). The residue in the flask, consisting of water, protein picrates, fat and picric acid crystals, was transferred to a glass beaker and subsequently filtered. The filtrate was discarded and the residue washed repeatedly with ether. The washed picrates were then ground in a mortar with small amounts of alcoholic hydrochloric acid (25 c.c. 3NHCl plus 75 c.c. absolute alcohol, unless otherwise stated in the results) and filtered. This process of grinding and filtration was repeated a second and third time. The combined filtrates (or supernatant fluid when centrifugation was used) measuring about 50 cc. were added to approximately 1,000 c.c. of

/dry

dry acetone. As soon as the precipitate of hydrochloride had settled the supernatant fluid was decanted and the residue passed through a sintered glass filter. The hydrochloride was repeatedly washed first with acetone, then with acetone-ether and, finally, with dry ether. The filter with its contents was then placed in a desiccator for further drying, whereupon the hydrochloride was tubed, sealed and stored in a refrigerator at 4°C.

In all cases the dried hydrochloride was weighed and tested for activity as soon as possible after its preparation. Unless otherwise stated in the results, not more than three days elapsed between the preparation of the hydrochloride and its testing. The method of testing was done as described in Part I of this work. To check the accuracy of this method, occasional full assays were carried out according to Trevan's mouse method (17, 73, 92).

(d) Results.

1. Time factor in fixation.

The effect of the lapse of time from the mincing of the pancreas until the beginning of extraction has been studied. The material was obtained and minced with picric acid, as previously described, weighed off in 200 gm., samples and the water content estimated and determined. The picrated samples were then stored in a refrigerator for varying periods as indicated until the extraction process was started. The procedure was similar for all samples except those indicated by an asterisk, where either the storage took place at room temperature or the acetone concentration was varied, these variations to be considered in succeeding sections. The summarised results are given in the following Table 24.

/TABLE 24.

TABLE 24.

Beef Pancreas.

Serial Number of Sample.	Period of fixation with picric acid.	Insulin yield per kilo of wet pancreas.
124	1 hour	2823
125	5½ hours	3225
126	24 hours	2480
127	2 days	2493
128	5 days	3285
129	35 days	2440
146 *	12 days	2737
107 *	13 days	3425
111 *	21 days	2550
120 *	26 days	2175
112 *	28 days	(2632 (2457 **
114 *	32 days	3286

** As determined by mouse assay.

2. Effect of Temperature on Picrated Mass.

It was subsequently necessary to determine the effect of temperature on the samples during the period of storage. Some samples were, therefore, stored at room temperature and others at refrigerator temperature. Growth of moulds on the samples stored at room temperature for long periods was particularly troublesome, so that, as indicated, tricresol or thymol was added to these. Material was collected and prepared as previously described. The samples were from the same batch and received similar treatment, except those indicated by an asterisk which belonged to different batches and received varied treatment. The summarised results are given in Table 25 on the following page.

In Sample No.142 the open specimen jar was placed in an incubating oven and left for two days, during which time all the fluid evaporated. The absolutely dry picrated mass that remained was placed in a dark cupboard for 10 days and subsequently extracted as the other samples.

3. Effect of Acetone on Picrate.

The next question that required settlemtnt was the possible effect of the aqueous acetone on the picrate. As has been indicated previously, the picrated mass is extracted 3 times in succession with aqueous acetone, during which process the insulin picrate, together with other inactive protein picrates, goes into solution. In view of the untoward effect of acid alcohol on insulin discussed elsewhere, it was thought necessary to determine the possible effect of acetone on the insulin picrate. The material was collected and treated as previously described. Unless otherwise indicated, the samples were all from the

/same

TABLE 25.

Beef Pancreas.

Serial Number of Sample	Period of fixation with picric acid.	Method of storage of sample	Insulin yield (I.U) per kilo of wet pancreas.
135	24 hours	On shelf at room temperature with tricresol added.	2350
136	4 days	In dark cupboard at room temp. (23°C) with tricresol added.	2500
137	6 days	In incubating oven at 40°C with tricresol added.	875.
138	7 days	In dark cupboard at room temp.(25°C) with tricresol added	2946 2815 ✳
139	15 days	In dark cupboard at room temp.(23°C) with tricresol added.	2595
142 ✝	2 days 10 days	In embedding oven at 60°C Dried material in dark cupboard at room temp.	2466
107 ✝	13 days	In refrigerator at 4°C	3425
113 ✝	21 days	On shelf at room temp. with thymol added	1920
111 ✝	21 days	In refrigerator at 4°C with thymol added	2550
120 ✝	26 days	In refrigerator at 4°C	2175
110 ✝	27 days	In refrigerator at 4°C	2958
112 ✝	28 days	In dark cupboard at room temp. with thymol added	2632 2457 ✳

✳ As determined by mouse assay.

114 ✝	32 days	In refrigerator at 4°C with thymol added.	3286
115 ✝	54 days	On shelf at room temp. with thymol added	-

same batch and received similar treatment. However, as is indicated in the following Table 26, the duration of the extraction period, i.e., the entire period that the aqueous acetone was in contact with the protein picrates has been varied.

TABLE 26.

Beef Pancreas.

Serial Number of Sample.	Total duration of extraction with acetone.	Insulin yield (I.U.) per kilo of wet pancreas.
130	1½ hours	3333
131	3 hours	3060
132	44 hours	2894
133	96 hours	420
134 ✝	2 hours	2575
129 ✝	3 hours	2440
107 ✝	15 hours	3425

4. Optimum Concentration of Acetone.

From the onset of this investigation of the picric acid-acetone method it became evident that this matter should receive full consideration. As claimed by its originators the specific solubility of insulin picrate in aqueous acetone forms the basis of the method. Through the action of picric acid all pancreatic proteins are converted to the respective protein picrates. From the mass of picrates the insulin picrate is selectively dissolved by aqueous acetone, the optimum concentration having been found to be 70 per cent.

In order to test this statement, the material was collected and treated as previously described. The water content was determined for the different samples and these

/were

were extracted with varying concentrations of acetone. In a particular batch the treatment was similar for all samples, so that differences in the yield are to be ascribed to the varying concentrations of acetone. Samples from other batches or samples having received different treatment are denoted by an asterisk. The results are indicated in the following Tables 27, 28:-

/TABLE 27.

TABLE 27.

Beef Pancreas.

Serial No. of Sample	Concentration of Acetone	Yield of Insulin hydrochloride in mg. per 200 gm. sample	Potency of hydrochloride I.U/mg.	Insulin yield (I.U) per kilo of wet pancreas.
148	40	0.058	-	-
149	50	0.240	1.16	1392
150	60	0.388	1.12	2172
151	70	6.577	1.10	3174
152	80	0.670	1.14	3819
153	90	0.328	1.14	1870
117	40	0.121	-	-
118	45	0.189	0.61	567
119	50	0.265	1.12	1484
120	55	0.275	1.58	2175
120(a)	60	0.343	1.36	2332
121	65	0.448	1.21	2710
121(a)	70	0.524	1.20	3144
122	75	0.677	1.17	3960
122(a)	80	0693	1.18	4088
123	85	0.474	1.20	2844
123(a)	90	0.281	1.36	1910
113 ⚜	60	0.400	0.96	1920
112 ⚜	60	0.351	(1.50 (1.40	(2632 (2457 ✳
125 ⚜	65	0.465	1.38	3225
134 ⚜	65	0.515	1.00	2575
109 ⚜	85	0.282	1.22	1720

✳ As determined by mouse assay.

/TABLE 28:

TABLE 28:

Sperm Whale Pancreas.

Serial Number of Sample	Concentration of Acetone	Yield of insulin hydrochloride in mg. per 200 gm. sample.	Potency of hydrochloride I.U./mg.	Insulin yield (I.U.) per kilo of wet pancreas.
5 (i)	55	0.223	1.60	1781
(ii)	60	0.241	1.12	1350
(iii)	65	0.470	0.78	1833
(iv)	70	0.571	0.78	2226
(v)	75	0.518	0.88	2279
(vi)	80	0.502	0.98	2460
(vii)	85	0.536	1.06 1.05	2840 2814 ✻
(viii)	90	0.210	1.40	1470
6 (i)	50	0.130	1.30	845
(ii)	55	0.281	1.12	1575
(iii)	60	0.467	0.86	2008
(iv)	65	0.570	0.78	2223
(v)	70	0.614	0.82	2517
(vi)	75	0.617	0.92 0.84	2838 2592 ✻
(vii)	80	0.684	0.92	3146
(viii)	85	0.675	0.94	3172

✻ As determined by mouse assay.

5. PRessure and Temperature during Distillation.

After extraction and subsequent filtration of the aqueous acetone, the acetone is evaporated from the latter. In all, the extracted samples this was done at reduced pressure with the temperature in the distilling flask not exceeding 30°C. However, Dickens and Dodds (83) had claimed that this step can be performed at ordinary pressure from a water bath. In order to test this statement, two samples, Nos. 134 and 134(a), received similar treatment with the exception that, whereas No.134 was distilled in vacuo at below 30°C., No.134(a) was distilled at ordinary pressure, the maximum temperature in the water bath having registered 87°C. and in the distilling flask 67°C. The duration of the distillation was approximately 2½ hours. Sample 134 yielded 2575 units per kilo of wet pancreas whereas the corresponding yield for Sample 134(a) was 2468 units.

6. Conversion of Picrate to Hydrochloride.

As Dudley (79) pointed out, this is effected by treatment of the picrate with a mixture of 75 cc. absolute alcohol and 25 cc. 3NHCl, the variables in the mixture being (i) the concentration of alcohol and (ii) the acidity. The underlying question in this statement is the role of the alcohol and acidity in a further selective isolation of insulin picrate from the accompanying inactive picrates. This was tested in two series of experiments, in the first of which the alcohol concentration was varied and in the second the acidity. Samples 140, 141 and 143 received similar treatment as regards time of fixation and extraction with acetone. However, with conversion of the picrate to the hydrochloride the alchhol concentration in each sample was varied.

In Sample 140 the combined picrates were ground up with 90 per cent alcohol in 0.75NHcl, and the hydrochloride precipitated. The undissolved residue was then treated with 75 per cent alcohol in 0.75 NHcl and the hydrochloride similarly precipitated. A similar procedure was followed with Sample 141, the picrate first having been ground up with 60 per cent alcohol in 0.75 NHcl and the residue with 75 per cent alcohol in 0.75 NHcl. With Sample 143 the picrate was ground with 95 per cent alcohol in 0.5 NHcl, the residue with 85 per cent alcohol in 0.75 NHcl, the residue of this with 75 per cent alcohol in 0.75 NHcl and the final residue with 65 per cent alcohol in 0.75 NHcl. The results of this series are given in the following Table 28:

TABLE 28.(a)

Beef Pancreas.

Serial Number of Sample	Treatment of Picrate	Yield of Insulin hydrochloride in gm/200 gm. sample	Potency of hydrochloride I.U/mg.	Insulin Yield (I.U) per kilo of wet pancrea
140				
Fraction A	90 per cent alcohol in 0.75 NHcl	0.336	1.80	3024
Fraction B	75 per cent alcohol in 0.75 NHcl	0.191	1.12	1069
TOTAL		0.527		4093
141				
Fraction A	60 per cent alcohol in 0.75 NHcl	0.634	1.20	3856
Fraction B	75 per cent alcohol in 0.75 NHcl			
TOTAL		0.634		3856
143				
Fraction A	95 per cent alcohol in 0.5 NHcl 85	0.140	0.84	588
Fraction B	per cent alcohol in 0.75 NHcl	0.333	1.28	2131
Fraction C	75 per cent alcohol in	0.251	0.70	878
Fraction D	0.75 NHcl 65 % alcohol in 0.75 NHcl	-	-	-
TOTAL		0.724		3597

In the second series the alcohol concentration was kept constant while the acidity was varied, as indicated in Table 29:-

TABLE 29.

Beef Pancreas.

Serial Number of Sample	Treatment of picrate	Yield of hydrochloride in gm/ 200 gm sample	Potency of hydrochloride I.U/mg.	Insulin Yield (I.U.) per kilo of wet pancreas.
144	75 per cent alcohol in 0.5 NHcl	6.666	0.96	3096
145	75 per cent alcohol in 0.3 NHcl	0.621	1.18 1.17	3662 3632 *
146	75 per cent alcohol in 0.2 NHcl	0.595	1.06	3153
147	75 per cent alcohol in NHcl 1.5	0.615	1.06	3260

* As determined by mouse assay.

7. Stability of Hydrochloride.

In all samples the hydrochloride was tested for potency as soon as possible after extraction. However, this was not always possible on the day following extraction and not infrequently samples had to stand over for longer periods before being tested. During this period the hydrochloride, which had previously been dried in a desiccator and sealed in a glass tube without special precaution, was kept in a refrigerator at 4°C. It was, therefore, thought desirable to test the stability of the hydrochloride under such conditions, the results being given in Table 30:-

TABLE 30.

Beef Pancreas.

Serial No. of Sample	Date of Test	Potency of hydrochloride I.U/mg.	Insulin yield (I.U.) per kilo of wet pancreas.
143 B	23/3/50	1.28	2131
	4/4/50	1.26	2097
	17/4/50	0.88	1464
145	24/3/50	1.18	3662
	10/4/50	0.98	3042
	18/4/50	0.74	2298
147	29/3/50	1.06	3260
	6/4/50	1.08	3321
	29/4/50	0.82	2522

8. Purification of Hydrochloride.

The hydrochloride as obtained by conversion of the picrate is obviously a crude and impure product. Attempts at purification of this, using some of the standard methods, were, therefore, considered. Dudley (61) had stated that, if iso-electric precipitation is carried out with a 2 per cent solution of the hydrochloride, a precipitate formed at pH5.7, carrying with it about 50 per cent of the activity and having a potency of 0.3 milligram = rabbit unit. Boivin and Guillemet (93) extracted insulin with acid alcohol, precipitated the active fraction by half saturation with ammonium sulphate and purified the extract according to Dudley's picrate method (61). By repeating the process of picrate precipitation and conversion of the latter to the hydrochloride, they obtained an active material with a potency varying from 5 to 10 units (International) per milligram. From this they

/separated

separated the active fraction by iso-electric precipitation at pH5. The precipitate was re-dissolved, inactive proteins precipitated at pH7.4 and 3.6 and the insulin again iso-electrically precipitated at pH5.4, thus obtaining an insulin assaying at 40 units per milligram. Moloney and Findlay (49) adsorbed insulin on charcoal from a solution of the hydrochloride; adsorbed impurities were removed by washing with an alcoholic solution of acetic acid and the insulin by digestion at normal temperature with a solution of benzoic or salicylic acid in 60 per cent alcohol, and recovered by evaporating the alcohol and extracting the benzoic or salicylic acid with ether. They thus obtained a highly purified insulin.

In an attempt to purify the hydrochloride obtained by picric acid-acetone extraction, the following procedures were used:-

(i) Sample 154 was extracted according to the general method and the picrate purified according to Dodds and Dickens (78). This consists of dissolving the picrate in N/10 sodium carbonate, precipitating by the addition of N/10 Hcl and conversion of the purified picrate to the hydrochloride. The yield obtained was 0.276 gr. and the activity 2.12 units per milligram, the total yield per kilo of wet panoreas thus being 2,920 units.

(ii) The hydrochloride, obtained from previously extracted samples, was pooled, the total weight being 2.0 gm. and the activity, as previously determined, 1.02 units per milligram. This was dissolved in distilled water and the pH carried to 5 by the dropwise addition of dilute sodium hydroxide. No precipitate was obtained within the acid range from pH2 to pH7, but on adjusting the pH to 8 a copious precipitate formed. This showed no activity on testing and the mother liquor was again brought to the acid side with dilute hydrochloric acid but without

/success

success of precipitation within the mentioned range.

(iii) 10 gm. of hydrochloride with a previously determined activity of 0.92 units per milligram were similarly collected as in (ii). This was dissolved to a concentration of 2 per cent in distilled water, the pH of the solution having been 4.2. This was carried to pH8 with sodium hydroxide and the precipitate removed. To the clear filtrate (pH2) sodium chloride was added to a concentration of 15 per cent. The brine, on testing, contained no activity. The precipitate was re-dissolved at pH2.2 and the reaction carried towards 5. Again, no precipitation was obtained within the acid range pH2 to pH7.

(iv) 16 gm. of hydrochloride was prepared from whale pancreas - activity 0.94 units per milligram. This was dissolved in 2000 mils. distilled water and the mixture made ammoniacal (pH8.1). The precipitate was removed by centrifuge, the supernatant fluid acidified to pH2.2 with sulphuric acid and 25 per cent sodium chloride added. The brine contained no activity; the precipitate was removed by filtration and re-dissolved in 1,200 mils. distilled water acidified to pH2 with hydrochloric acid. To this was added sodium chloride to a concentration of 15 per cent; the brine showed no activity and the precipitate, separated by filtration, re-dissolved in 350 mils. water acidified to pH2 with hydrochloric acid. No precipitate formed within the iso-electric range (pH5-5.4) but, on carrying the reaction to pH8.8-9, a further precipitate came down. The pH of the mother liquor was adjusted to 5.4, again without success. On testing, both the mother liquor and the pH8.8-9 precipitate showed activity. This was re-dissolved, added to the mother liquor and the pH adjusted to 2. The solution was now salted out with 9 per cent sodium chloride; the brine

/showed

showed no activity and the precipitate re-dissolved at pH2; again, no precipitate was obtained within the iso-electric range. Finally, the solution (at pH2) was salted out with 5 per cent sodium chloride and the brine, showing no activity, discarded. The precipitate was dissolved in 50 mils.N/10 Hcl and dialised against distilled water. To this was then added 20 times its volume of alcohol-ether and the precipitate collected by centrifugation. This was dried in an incubating oven at 37°C. The yield of insulin thus obtained was 0.444 gm. and the activity 9.80 units per milligram.

(v) 5 kilos of beef pancreas were extracted according to the method as it was finally adopted. The total yield of hydrochloride was 16.425 gm. and its activity 1.22 units per milligram, giving a yield of 4008 units per kilo of wet pancreas. This was dissolved in 1000 mils. of water, the pH adjusted to 2.2 with dilute hydrochloric acid and sodium chloride to a concentration of 25 per cent added. The precipitate was dissolved in 300 mils. of water, the mixture acidified to pH1.8 with hydrochloric acid and salted out with 15 per cent sodium chloride. The precipitate was dissolved in 150 mils. of water, acidified to pH2.0 with hydrochloric acid. To this solution dilute sodium hydroxide was added slowly and the acidity adjusted to pH5. To this was now added 150 mils. of a sodium acetate buffer pH5.4 and the pH gradually increased until precipitation seemed at a maximum (pH5.4-5.5). This was left at refrigerator temperature for 48 hours and then filtered. The iso-electric precipitate was dissolved in 75 mils. of water containing 0.85 mils.glacial acetic acid and the procedure for the preparation of crystalline insulin further followed as described by Romans, Scott and Fisher (77). The weight of crystalline insulin obtained was 0.592 gm. assaying at 20.02 units per milligram.

/(e) Discussion.

(c) Discussion.

The most outstanding feature of the method is the stability of the picrate after mincing the original material with an adequate amount of picric acid crystals. However, the amount of picric acid, as advised by Dickens and Dodds, is considered too low for complete fixation, especially if the material has to stand for some length of time before being worked up. During the initial stages of this investigation it was experienced on several occasions that minced samples had to be discarded due to the low yield obtained from them. The cause of this was traced to insufficiency of picric acid, resulting in the partial or total loss of, not only the active principle, but also the accompanying inactive hydrochlorides. It is advised that the amount of picric acid crystals, previously well drained on a Buchner funnel, be not less than 80 gm. per kilo of material.

Dudley's claim for inessentiality of refrigeration after adequate fixation with picric acid (79) has been amply substantiated. As indicated by the results given in Table 24, no appreciable differences can be detected in the insulin yield of samples that had been stored for varying periods of from 1 hour to 35 days. Smaller differences can be ascribed either to sample variation or differing procedures during the extraction process.

The results showing the effect of temperature on the picrated mass are even more informative. Samples that had been kept at refrigerator temperature (4°C) for a maximum time period tested of 32 days show no decrease of insulin yield on extraction. For samples stored at room temperature it was found necessary to add tricresol or thymol to the picrated mass in order to prevent the growth of moulds. In none of these untoward effects of either

/tricresol

tricresol or thymol are indicated. Neither is the yield appreciably affected by storage at room temperature, the insulin content of samples having been thus stored for periods of 4, 7, 15, 21 and 28 days, comparing favourably with those that had been stored at refrigerator temperature or extracted shortly after fixation (c.f.samples 124 and 125, Table 24). Neither does storage in light or darkness seem to have effect on the picrate. Sample 113 (Table 25) would indicate a gradual decrease of insulin content when stored in light as compared to Sample 112 (stored in darkness). Both these samples were extracted with 60 per cent acetone (Table 27) and the results indicating the difference in yield are not sufficiently convincing to warrant the statement that light has a detrimental effect on the stored picrate.

Sample 115 (Table 25) yielded no insulinhydrochloride. This may be due partly to the low acetone concentration of the extracting medium - 50 per cent. However, both samples 149 and 119 (Table 27) and sample 6(i) (Table 28) had been extracted with this low concentration of acetone and had still given approximately half the normal yield. Sample 149 was stored for 6 days at refrigerator temperature prior to extraction and sample 119 for 26 days at refrigerator temperature. It, therefore, seems plausible to assume that the loss of insulin in Sample 115 is the effect of the long period of storage (54 days).

The results as shown in Tables 24 and 25, therefore, indicate that the well-mixed and sufficiently picrated mass, should the circumstances demand it, can be stored at either refrigerator or room temperature for a period of at least 20 to 30 days, before being worked up without serious loss of insulin. The importance of this fact, as far as the collection of pancreas from isolated areas for either

/commercial

commercial production or research purposes is concerned, is so obvious that it needs no further comment.

However, prolonged exposure of the picrate to higher temperature results in serious loss of activity. This is shown by sample 137 (Table 26) which had been incubated in an oven at 40°C for 6 days. This sample had been extracted with 65 per cent acetone, the yield being 0.393 gm. hydrochloride, which compares favourably with the yields obtained from other samples extracted with this concentration (c.f. samples 121, 125 and 134 - Table 27). However, the activity of the hydrochloride in sample 137 was only 0.44 units per milligram as compared to 1.21, 1.38, 1.00 units per milligram in samples 121, 125 and 134 respectively.

In contrast to this is the result of sample 142. This sample was placed in an embedding oven at 60°C for two days (in an open jar, the jar in sample 137 having been kept closed). During this time all the water evaporated leaving the picrated mass hard and dry. This was placed in a dark cupboard for a further period of 10 days before being tested and still gave a yield well within the normal range (2466 units per kilo of wet pancreas). In order to account for the loss of activity in sample 137 as compared to sample 142, it will be necessary to look further afield than the effect of temperature over a longer period. Considering that the higher temperature of 60°C for a period of two days had no or little effect on the insulin content of the sample, it is difficult to visualise that the lower temperature of 40°C over a period of six days could result in so much loss of activity. It seems probable that other factors as the length of time, and especially unrecognised factors of the fluid medium (as in sample 137), contributed more to loss of activity than higher temperature alone.

/But

But sample 137 does indicate that if the picrated mass is stored at room temperature, this should be in a cool place away from the sun. Similarly, if the material is to be transported, the containers with the picrated mass plus separated water should be kept in as cool a place as possible, as exposure to heat (in a non-insulated truck of a train for instance) would result in serious loss of active material.

The observation on sample 142 can be of great practical importance. Dehydration of the picrated mass results in a 70 per cent loss of weight of the original material (the water content of a 200 gm. sample of minced picrated pancreas has been found to average 140 mils). This not only renders transfer easier but also cheaper.

Working on a small scale, separation of the minced material from the extracting medium has been effected by filtration. This process was often found to be very slow and tedious and, in isolated cases, almost impossible. An additional advantage of the dried picrate is that the added extracting fluid is separated from the solid material without the least difficulty by filtration. On a small scale dehydration is accomplished within a few hours by placing the material in an open, flat-bottomed dish in an embedding oven at 60°C. On a larger scale this would probably be done in an easier and more effective manner.

As regards the effect of acetone on the picrate, Table 26 shows that over-exposure results in almost total destruction of the insulin. A similar effect of acetone on crystalline insulin has been postulated by Scott and Fisher (94); this is enhanced by the presence of small amounts of hydrochloric acid. To a maximum of 44 hours only slight effect is indicated, but exposure of the picrate to the aqueous acetone for 96 hours (sample 133)

/caused

caused almost complete destruction of the insulin. In the series 130-133 there is, first, a gradual and then a sudden decline of insulin content of the samples. The differences between the insulin yield in samples 130, 131 and 132, are comparatively small and insufficient to warrant a direct conclusion. However, it would seem advisable, when extracting the picrated mass with aqueous acetone, to make this procedure as short as possible. As sample 130 indicates, a total extraction period of 1½ hours is sufficient - ½ hour for each of three extractions. Evaporation of the acetone from the filtered extracting fluid as soon as possible is strongly suggested. As already indicated, the extracting fluid is separated from the minced material by filtration. It was constantly found that the clear filtrate deposited on standing, a fine inactive precipitate increasing in amount with time.

The claim of Dickens and Dodds that the solubility of insulin picrate in mixtures of acetone and water is (i) sharply defined and (ii) at an optimum of 70 per cent acetone (83) has not been substantiated. The authors stated that "the optimum concentration of acetone for the solubility of insulin picrate was found to be 70 per cent. Above and below this concentration the solubility decreased markedly."

Tables 27 and 28 present the results obtained when samples were extracted with acetone concentrations ranging from 40 to 90 per cent. In all these cases the water content of each batch of samples was determined as previously described and calculations based thereon. Dickens and Dodds give no account of their determination of the water content but state that the water which separated during the mincing process was pressed out and

/a

a volume of dry acetone equal in weight to the residue added to the latter. Such a procedure where (i) the amount of water separating during the mincing varies (with frozen pancreas very little water separates) and (ii) the amount of water pressed out is uncontrolled, is naturally open to inaccuracies as far as the first extraction is concerned. This would, naturally, affect the ultimate result, notwithstanding the fact that the second and third extractions are performed with the required concentrations of acetone. Whereas Dickens and Dodds had postulated an optimum acetone concentration of 70 per cent, the results of Tables 27 and 28 clearly indicate that, with accurately-calculated values, the optimum acetone concentration is much closer to 80 than to 70 per cent. In the first series (Table 27) sample 151 (70 per cent) yielded 3174 units as compared to 3819 units of sample 152 (80 per cent). In the second series the respective values are 3144 and 4088 units for samples 121(a) and 122(a). In this series additional information is provided by sample 122 (75 per cent) in which the yield almost equalled that of sample 122(a). The difference in yield between the 70 per cent and 80 per cent extractions in the first series is 645 units and in the second series 944. Although not very appreciable, these differences are sufficient to be of practical importance. Unfortunately, no extractions were made with 75 and 85 per cent acetone concentrations in the first series but sample 123 in the second series unmistakably indicates a marked decrease in yield with an 85 per cent acetone concentration. This is further evidenced by the comparatively low yield of sample 109 (1720 units) which had also been extracted with 85 per cent acetone. As far as beef pancreas is concerned, the optimum acetone concentration undoubtedly lies between 75 and 80 per cent.

In dealing with whale pancreas (Table 28) similar conclusions are arrived at, with the exception that the optimum value has shifted to the 80 to 85 per cent level. In both series (Table 28) the maximum yield was given by 85 per cent extractions. However, the differences between 80 and 85 per cent extractions in these two series are but slight. In the first series the difference between the 70 per cent extraction and maximum yield (samples 8(iv) and 8(vii) is 614 units and the corresponding figure in the second series (samples 6(v) and 6(viii) 655 units. In all these (Tables 27 and 28) extraction with 80 per cent acetone for beef pancreas and 80 to 85 per cent for whale pancreas indicates an increased yield of approximately 20 to 25 per cent over a 70 per cent extraction. For whale pancreas 90 per cent extraction (sample 8(viii) shows a similar marked decrease, as was found for 85 per cent in beef.

The possibility of slight variations in optimum concentration for pancreas from different sources is indicated by a comparison of beef and whale pancreas. The optimum concentration was not determined for pig and sheep but it would seem advisable to determine this for pancreas from a particular source prior to extraction of the material on larger scale.

As regards the "sharply defined" solubility of picrate and its "marked decrease" above or below the stated 70 per cent level, the evidence is not very conclusive. Tables 27 and 28 indicate that the picrate is soluble to greater or lesser extent in concentrations of acetone varying from 50 to 90 per cent. In the first series (Tables 28) the total yield obtained from 50 and 90 per cent extractions constitute about 30 to 50 per cent respectively of the maximal yield. A similar proportion is indicated for the second series of Table 27. The difference in solubility is

/even

even less marked when considering the results of Table 28. However, it is clear from these results that the solubility increases with increasing concentrations until the optimum level is reached, and then decreases less gradually or more markedly. This is also reflected by the actual yield of hydrochloride which, in the second series (Table 27) rises gradually from 0.265 gm. for a 50 per cent extraction to a maximum of 0.693 gm. for the 80 per cent, then decreasing again to 0.281 gm. for the 90 per cent. This indicates that the solubility of the picrates is more of a quantitative than of a qualitative nature. The potency of the converted hydrochloride remains unaffected to a large extent throughout the range of concentrations varying only within narrow limits (1.12 to 1.58 units per milligram in the second series of Table 27). Added to this is the fact that in none of the series is the most potent material extracted by the optimum concentration. In the four series of Tables 27 and 28 the potency of the hydrochloride obtained from optimum concentration extraction is 1.14, 1.18, 1.06, 0.94 units per milligram as compared to 1.16, 1.58, 1.60, 1.30 units per milligram respectively, representing the most potent final products of the different series. In most cases extraction with 50 or 90 per cent of acetone, although giving a smaller yield, gives a purer product than extraction with the optimum concentration. If the solubility of acetone for insulin picrate had been selective and specific, it would be expected that the optimum concentration would yield a product surpassing in potency those products obtained by extractions with concentrations other than the optimum.

Additional support for this statement is furnished by the following observation: The picrate obtained by

/Extraction

extraction with 50 or 90 per cent acetone is of small quantity and when treated with acid alcohol for conversion to the hydrochloride, dissolves completely or almost so in the alcohol, so that very little undissolved picrate remains. As the acetone concentration is increased to 55, 60, 65, etc. per cent, so the quantity of picrate increases and likewise the amount of picrate undissolved after treatment with acid alcohol increases. With 50 per cent acetone extraction, the weight of dried picrate obtained after evaporation of the acetone has consistently been found to vary from 0.4 to 0.6 gm. When treated with acid alcohol this either dissolves completely or a negligible residue is left. With 80 per cent acetone extraction the dried picrate weighs in the neighbourhood of from 3 to 4 gm., of which not more than 0.75 to 1 gm. goes into solution in the acid alcohol, leaving the bulk of protein picrates (2 to 3 gm.) undissolved. It seems as if selective solution of insulin picrate is more a feature of the acid alcohol than of the acetone.

This raises the interesting question whether acid alcohol (or alcohol only) cannot substitute acetone in the extraction process. This being more of theoretical than practical importance, the matter was not further investigated as (granted it is the case) it would not add to the effectiveness or practicability of the method in general. Judging from the above observations, there seems no reason why alcohol should not effectively displace acetone in the extraction.

The picrate which separates when the acetone is evaporated, apparently consists of a minor fraction of insulin picrate accompanied by a major fraction of physiologically inactive protein picrates. The specificity of solubility of acetone is probably related more to the entire complex than to insulin picrate in particular, thence

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the disproportionate increase of picrate to hydrochloride with increasing concentrations of acetone. As this concentration is increased, more of the entire complex goes into solution, carrying with it also insulin picrate. As far as specificity is concerned, it can safely be stated that the very low and very high acetone concentrations are just as specific, if not more, for insulin picrate than the optimum concentration. This is obviously due to the partial exclusion of much inert material by the low and high concentrations of acetone. This would also account for the greater potency of the hydrochloride obtained from levels above and below the optimum. The fact that so much picrate that had dissolved in theaqueous acetone at the optimum level is insoluble in the acid alcohol when treated with this, clearly proves that the alcohol has a greater selectivity and specificity for insulin picrate than acetone.

However, notwithstanding this apparent ineffectiveness of aqueous acetone in selectively dissolving insulin picrate, there can be no doubt that it completely removes the active principle from the picrated mass. This is proved by the high yields of insulin obtained by this method as compared to others. A direct comparison in terms of, say, crystalline insulin between the two methods makes it obvious that the yield obtained by the picric-acid acetone method surpasses that of the acid alcohol method (see later).

The observation of Dickens and Dodds (83) that the acetone can be distilled off from the aqueous mixture at ordinary pressure from a water bath has been confirmed. However, this is of more theoretical than practical interest as distillation at ordinary pressure is a much slower process than at reduced preesure. This fact consequently has no practical advantages in increasing the effectiveness

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of the method. It does, however, emphasize again the stability of the picrate to higher temperatures.

Concerning the conversion of the picrate to the hydrochloride, the variable factors are (i) the concentration of alcohol and (ii) the acidity of the mixture. As has been pointed out in the preceding paragraphs, the action of the aqueous acetone is the solution of the complex of protein picrates which accompany insulin picrate. Selective solution of insulin picrate from the accompanying picrates is more a feature of the acid alcohol. The ultimate hydrochloride is still a very crude product and the results of Tables 28 and 29 represent attempts at increasing the specific solubility of acid alcohol for insulin picrate by varying the alcohol concentration and acidity.

Sample 141 (Table 28(ω)) shows that 65 per cent alcohol (in 0.75 NHcl) is as effective in dissolving the picrates and converting them to the hydrochlorides as 75 per cent alcohol (as advised by Dudley (80). The total yield of hydrochloride (3856 units per kilo) and its activity (1.20 units per mg).does not differ in any respect from the yield and activity as obtained by the use of 75 per cent alcohol. That solution of the active principle is quantitative with this concentration of alcohol, is evidenced by the further result of the same sample where subsequent treatment of the residue with 75 per cent alcohol yields no further hydrochloride - either active or inactive. Where the picrates were treated, first with 90 per cent alcohol (in 0.75NHcl - sample 140, Table 28), the resulting yield of hydrochloride (Fraction A) is somewhat less (0.336 gm.) and its activity slightly increased (1.80 units per mg.). However, subsequent extraction of the residue with 75 per cent alcohol (in 0.75 NHcl) had given a further hydrochloride (Fraction B) of slightly lesser activity. This proves that a 90 per cent concentration of alcohol does not completely

/remove

remove the insulin from the mixture of picrates. Similarly, further fractionation (sample 143, Table 28) is obtained by treatment of the mixture of picrates first with 95, then 85, 75 and 65 per cent of alcohol (in 0.75 NHcl). The last of these, yielding no further hydrochloride (there is no Fraction D) indicates that a concentration of 75 per cent is effective for the complete removal of insulin picrate. From this it is to be concluded that concentrations of alcohol (in 0.75NHcl) ranging from 65 to 75 per cent (concentrations lower than 65 not having been tested) are effective in quantitatively removing insulin picrate from the mixture. Use of higher concentrations of alcohol results in only the partial removal of the active principle from the mixture (Fraction A, sample 140 and Fractions A and B sample 143). Moreover, it is evident that no increase in activity or purification can be accomplished by varying the concentration of alcohol used for conversion of the picrate to the hydrochloride.

This phenomenon of fractionation of insulin was encountered elsewhere in this work. In an attempt to obtain a purer and more potent hydrochloride, the picrate was washed first with (i) 96 per cent alcohol (without acid)and second with (ii) 96 per cent acetone (without acid) and finally with (iii) 75 per cent alcohol in 0.75 NHcl for conversion to the hydrochloride. The yield after (iii) was so small without a corresponding increase in activity that the washings of (i) and (ii) were investigated, which revealed that amounts of picrate carrying active principle had dissolved in the high concentrations of alcohol and ether. These were precipitated and converted to the corresponding hydrochlorides. Thus three fractions of hydrochloride were obtained:

(i) Fraction A - the picrate soluble in 96 per cent alcohol;

/(ii)

(ii) Fraction B - the picrate insoluble in 96 per cent alcohol but soluble in 96 per cent acetone;

(iii) Fraction C - the picrate insoluble in either 96 per cent alcohol or acetone but soluble in 75 per cent alcohol.

These three fractions, on testing, showed the following activity:

Fraction A - 2.25 units per mg.

Fraction B - 1.84 " " "

Fraction C - 1.20 " " "

This fractionation of the insulin is probably the result of the specific solubility properties of the protein fission products in the various solvents.

Turning to the variation of the acidity of the alcoholic hydrochloric acid, the results of Table 29 indicate that, within the limits tested (75 per cent alcohol in 0.2 - 1.5 NHcl) acidity does not affect the conversion of the picrate to the hydrochloride; neither does it increase or decrease the solubility of insulin picrate. The yields in samples 144-147, and the potency of the hydrochloride are not affected by variations of the acidity. Increased selectivity can, therefore, not be obtained by variation of the alcohol concentration or acidity.

Dudley (79) extracting insulin from fish islet tissue claimed an activity of 1 milligram = 1 rabbit unit for the crude hydrochloride, whereas Dickens and Dodds (82) found an activity varying from 1 to 0.25 milligrams = 1 rabbit unit.

In the series of extractions for this investigation the first claim has been amply confirmed. Except in extreme variations of procedure, the potency of the hydrochloride has remained remarkably constant, varying

/from

from 1.10 to 1.20 international units per milligram, lower and higher values obtained with certain samples being the result of such variations. However, the claim for a crude hydrochloride assaying at 4 to 6 international units per milligram has, with a series of 144 extractions according to this method, not been substantiated. In none of these, even with preliminary purification of the picrate, has it been possible to obtain a hydrochloride showing an activity of more than 2.5 international units per milligram. With all these extractions it has been the experience that, with similar procedure, the yield of hydrochloride and its potency agree within very narrow limits. The results of Dickens and Dodds, where a specific yield of hydrochloride has an activity of 1 unit per milligram and a second yield, obtained through identical procedure, an activity of 4 to 6 units per milligram are, therefore, the more difficult to explain. Notwithstanding all possible variations of the method that had been tried out, the exact procedure whereby a crude hydrochloride assaying at 4 to 6 units per milligram can be obtained, has not been struck. As far as the results from 144 extractions are concerned, it has been found impossible with standard procedure (such as described by Dickens and Dodds) to obtain a crude hydrochloride with a potency exceeding 1.80 to 2.00 units per milligram, the average value lying much closer to 1 than to 2.

That the hydrochloride is not very stable under ordinary conditions of storage is shown by the results quoted in Table 30. In all three samples 143B, 145 and 147, there is a gradual decrease in the potency of the hydrochloride with increasing storage period. Samples 143B and 147 indicate that, under conditions as described, it is stable for about 1 week, after which period

/it

it gradually loses potency. In sample 145 storage for 17 days caused a decreased activity of 0.20 units per milligram, and 0.44 units in 25 days. Samples 143B and 147 likewise show a decreased activity of 0.40 and 0.26 units per milligram when stored for 24 and 31 days respectively. Furthermore, it was repeatedly experienced that hydrochloride that had been stored for periods of 2 to 3 months had invariably lost all or most of its activity.

These results indicate that the hydrochloride should be tested for activity as soon as possible after its preparation, in any case not later than 1 week afterwards. Also, it should be purified as soon as possible after having been prepared. If it has to stand over for periods exceeding 1 week or where it has to be transported from an initial extracting plant elsewhere for purification, it would be advisable to store it under conditions ensuring greater stability such as complete drying, sealing over nitrogen and lower temperature.

From the result of sample 154 it is evident that purification of the picrate by this method could not lead to much success and it was realized that the only practical alternative method was iso-electric precipitation. Initially, however, much difficulty was experienced with this. In the first attempts the hydrochloride was obtained by pooling previously extracted samples which had been stored for varying periods. This was done before the instability of the crude hydrochloride under the specific conditions had been definitely shown, and failure to obtain iso-electric precipitation, must have been due to the inactivity of the original material.

In a later attempt (see (iv) page 122) hydrochloride was freshly prepared. Failure to obtain iso-electric precipitation in this instance was probably due to two

/factors

factors: (i) initial precipitation at pH8.1 and (ii) absence of a buffered solution during the process of iso-electric precipitation.

The initial precipitation at pH8.1 brought down a mass of material which, as a result of similar procedure in the acid alcohol extraction method, was considered inactive and discarded without having been tested for activity. In view of the fact that at a later stage an activity containing fraction was precipitated at pH8.8 - 9, it can be assumed that the first precipitate at pH8.1 carried down with it an appreciable amount of activity, which consequently was lost. Subsequent failure to obtain iso-electric precipitation can possibly be ascribed to the absence of a suitable buffer solution. As pointed out by Boivin and Guillemet (93), insulin is impurified by other proteins which behave so much like it, that it is impossible to separate these by precipitation with sodium chloride, ammonium sulphate, picric acid or alcohol. The presence of large quantities of these inactive insulin-like proteins, a corresponding smaller amount of active material and the absence of a buffered solution were probably the causative factors responsible for the failure to obtain iso-electric precipitation.

With a following effort (see (v) page 123) these causes were remedied and the active substance was precipitated at its iso-electric point without difficulty. The process of purification was continued further and crystalline insulin prepared. From the original ± 22,000 units almost 12,000 had been recovered in crystalline form. Working on a small scale, active material is lost at various stages; on a large scale these can be recovered and worked up so that the total yield will be increased. As it is, a yield of 2,370 units per kilo of wet pancreas was obtained. As a

/control

control (see later) beef pancreas was extracted with acid alcohol and crystalline insulin prepared according to the method of Romans, Scott and Fisher (loc.cit.). The yield obtained with this procedure was 1892 units per kilo of wet pancreas.

The method then, as it was finally adopted and as it is advised, can be summarized as follows:-

(i) Obtain the material as fresh as possible, remove excess fat, connective tissue, glandular structures, etc. and mince, adding picric acid crystals while this is in progress. The amount of picric acid should not be less than 80 gm. per kilo of material. After mincing, the entire mass should be re-minced a second and a third time to ensure even mixing.

(ii) The picrated mass should be stored preferably in glass or earthernware containers, the effect of other types such as metal, not having been tested out. If possible, storage could take place at refrigerator temperature, otherwise room temperature will suffice provided thymol or tricresol is added to the mixture in order to prevent mould growth. In the latter case samples may be stored in either light or darkness, taking care to keep the material as cool as possible. Under any of these conditions storage can take place for a period of from 20 to 30 days without serious loss of activity.

(iii) Immediately before extraction, determine the water content of the mixture from a small sample as previously described. Add to the mixture sufficient absolute acetone to make the concentration 75 to 80 per cent. If pancreas, other than beef is extracted, it would be well advised to determine the optimum acetone concentration for the

/specific

source as previously described. Re-extract the residue a second and a third time with 75 to 80 per cent acetone and pool the filtrates. Avoid prolonged contact of the acetone with the dissolved picrate; the sooner the acetone is evaporated the better. Extraction of the picrated mass for ½ hour is sufficient; under no circumstances must the acetone be left in contact with the picrate for longer than 24 or at the utmost 36 hours.

(iv) Evaporation of the acetone can be effected either in vacuo or at ordinary pressure from a water bath. In view, however, of the shorter time necessary for in vacuo distillation, this is recommended.

(v) Preliminary purification of the picrate is unnecessary as fractional precipitation with alcohol or treatment with sodium carbonate does not yield an appreciably purer substance. The picrate is converted to the hydrochloride by treatment with 65 to 75 per cent alcohol in hydrochloric acid which may vary from 1.5 - 0.2 N.

(vi) The hydrochloride is precipitated by adding to the alcoholic hydrochloric acid solution 10 to 20 times its volume of dry acetone.

(vii) It is advisable to test the hydrochloride or to purify it as soon as possible after its preparation, unless special precautions are being taken with regard to its storage.

(viii) Purification of the hormone can be effected by dissolving the hydrochloride in water, salting out first with 25 and then with 15 per cent sodium chloride, and finally precipitating the insulin at its iso-electric point pH5.4.

Other possible methods of purification have not been exploited. Indications were obtained that silica

/and

(and probably other piezzo-electrical substances) may advantageously be used for insulin purification by adsorption and it is proposed to continue with further investigations in this direction.

(f) Conclusions:

Reviewing the method as a whole, the following conclusions are arrived at:-

(i) The yield of crude hydrochloride obtained with this method is nearly double that of pure insulin, which is obtained with the acid alcohol method (3500 - 4000 units per kilo of wet pancreas). With purification of the hydrochloride on a small scale an appreciable amount of activity is lost; it could be expected that larger scale purification would result in less loss. A comparison of the two methods carried through to the crystalline product, showed a yield of 1892 units per kilo of wet beef pancreas for the acid alcohol method as compared to a yield of 2370 units per kilo for the picric acid acetone method.

In a laboratory the proportion, yield crude hydrochloride:yield crystalline insulin, can be determined in a series of experiments so that subsequent extractions, for research purposes, need be carried through only to the crude hydrochloride stage, from which value the amount of crystalline insulin obtainable can be calculated.

(ii) The solubility of aqueous acetone for insulin picrate is not specific; it is probably more specific for the entire complex of picrates behaving like insulin. In this respect alcohol, or probably other organic solvents, could be substituted for acetone. However, it is certain that all insulin activity is quantitatively removed from the picrated mass by this solvent.

/(iii)

(iii) Perhaps the most outstanding feature and merit of the method is the stability of the picrate to varying temperature. The advantages of this and its practical application are numerous. It allows the collection of material from distant sources (i.e., the ocean, uncivilized country, thinly-populated areas, etc.) where freezing facilities are not available. In addition to this, it allows the storage of such material for fairly long periods without refrigeration until such time that extraction can be undertaken.

An interesting observation that may lead to important developments is the stability of the picrate under conditions of dehydration of the minced picrated mass. The advantages are obvious: the bulk of material is very much reduced; this will require an equally much-reduced volume of extractant; transportation is simplified in many ways; the possibility of extraction of the dried, picrated mass with water, instead of an organic solvent looms in the foreground; the possibility of some other effective fixative replacing picric acid followed by dehydration and subsequent aqueous extraction cannot be ignored. The prospect of further research in this direction is being held out.

(iv) For experimental work and extraction on small scale, the method is to be recommended. As compared to other methods it gives a maximum yield with a minimum of precautions and limitations. It works easily and rapidly.

(v) A serious disadvantage of the method is the staining and fixing properties of the picric acid and its explosiveness when dry. Probably the greatest objection to it is its staining property; all objects in contact with it - apparatus, hands, etc. - become intensely stained, so that, on the whole, it is a messy method as compared

/to

to the acid alcohol. A possible injurious effect of it on human contacts (c.f. action of tannic acid) has as yet not been postulated. For this research it was handled for a period of over two years; as yet no untoward effect has been observed.

(vi) It is doubtful whether, in its present form, it can replace the acid alcohol method for large scale extraction. Apart from the stability of the picrate, it has nothing to offer above the acid alcohol method; added to this is the serious disadvantage mentioned in (v) above. As pancreases are at present collected only from large centres where there are ample refrigeration facilities, this advantage is not of much practical concern. If, however, at some future stage the necessity should arise for the collection of material from other sources, the method could assume greater importance. Added to this is the fact that full details regarding commercial extraction methods are not published; there may, therefore, be unpublished data which give the acid alcohol method a decided advantage over the picric acid acetone method.

III. EXTRACTION OF INSULIN FROM VARIOUS SOURCES.

Introduction.

Since the first successful extraction of insulin by Banting and Best and the subsequent improvements in the methods of extraction and purification, the hormone has been isolated from many and varied sources. However, lack of an accurate method of assay and uniformity in expressing activity during the first years, when most of the work was done, makes a direct comparison of the yields obtained by various authors from different sources a difficult matter. In addition, the end products obtained by different workers represented the hormone in varying degrees of purity, depending on the method of extraction and purification, which factor further complicates matters because, as has been shown, increased purification is accompanied by increased loss of active material.

In past years the pancreas from beeves and pigs has probably been the most commonly drawn-from source for research and experimental purposes. Apart from the earlier work of Banting and Best, the Toronto workers have almost exclusively used beef pancreas for their work on extraction and purification methods (43). Similarly, many other workers have found this source useful and convenient.

Best and Scott (43) reviewed the increasing yields of insulin obtained by them with improved methods. From a mere 15 units (modified Toronto) per kilo of beef pancreas in April, 1922, they successively increased the yield to 40, 90, 400 and 900 units per kilo in June, 1923, and conclude that "the increase in acidity of the extractive has been the greatest single factor in improving the yields". This yield was obtained with acid (acetic acid)

/alcohol

alcohol extraction, precipitation with ammonium sulphate and iso-electric precipitation. In the same year Murlin and co-workers (119) claimed yields of insulin amounting to 4,000 Toronto units per kilo of material. These were obtained by aqueous extraction with N/5 hydrochloric acid at 75°C. for one hour, followed by salting out with sodium chloride and iso-electric precipitation.

In 1924 Somogyi, Doisy and Shaffer (53), using their method of extraction with acid (sulphuric) alcohol, precipitation with ammonium sulphate and iso-electric precipitation (loc.cit.) obtained yields varying from 1,500 to 2,500 Toronto units per kilo of beef pancreas. In the same year Fenger and Wilson (120), using the above method, determined the insulin content of the pancreas from cattle, hogs and sheep and obtained a yield of 1,800 rabbit units per kilo of beef pancreas. Similarly, Moloney and Findlay (50), by purification of crude insulin through adsorption on charcoal, obtained 2,000 Toronto units per kilo of beef pancreas. Dickens and Dodds, applying the picric acid acetone method (83) to the extraction of insulin from various sources (78), obtained 2,500 rabbit units per kilo from ox pancreas.

In 1927 Blatherwick and his associates (54), using their method (loc.cit.), obtained from 1,800 to 2,500 clinical units per kilo of beef (assayed against Lilly's "iletin" as standard). After the third iso-electric precipitation, this was decreased to 1,000 to 1,400 clinical units per kilo. Jephcott(57), investigating the method of the Toronto workers with reference to the optimum alcohol and acid concentration for the extractant, obtained a yield of 3,000 international units for beef pancreas. Scott and Parker (55) obtained 2,000 international units from the same source. Starting with the fresh

/material

material and carrying the process through to the crystalline stage, Romans, Scott and Fisher (77) obtained yields varying from 1,800 to 2,000 units per kilo for beef pancreas. Fisher and Scott (121), investigating the insulin content of the pancreas in cattle of various ages, found the following values:

Foetal calves (under 5 months) ...	33,200	international units per kilo,
" " (5 - 7 months) ...	23,100	ditto
Calves (6-8 weeks on milk diet) ...	11,400	ditto
Cattle (2 years)	4,800	ditto
Cows (7 years and older, pregnant)	2,200	ditto
Cows (9 years and older)	1,800	ditto

Notwithstanding the lack of uniform expression of activity amongst earlier workers (c.f.rabbit unit, original Toronto unit, modified Toronto unit, clinical unit, international unit), it is clear that beef pancreas has been extensively studied and has yielded amounts of insulin varying from 2,000 to 3,000 international units per kilo.*

Best and Scott (43) state that "pork pancreas has consistently given us somewhat larger yields in experimental lots than has beef pancreas." In their studies on the picric acid acetone method (83), Dickens and Dodds have largely made use of pig's pancreas and have found values varying from 3,000 to 4,280 rabbit units per kilogram. Fenger and Wilson (120) obtained 1,800 rabbit units per kilo of pig's pancreas. Scott (122) has prepared crystalline insulin from pig's pancreas in amounts slightly exceeding the values obtained for beef pancreas.

Apart from these amounts of insulin extracted from beef and pig's pancreas, the following yields have been

/obtained

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* Toronto units in the above paragraphs refers to modified Toronto unit, ie., 1/3 of the original Toronto unit or rabbit unit.

obtained from other mammals:-

Sheep	-	1,000	rabbit	units/kg.	(78)
"	-	1,800	"	" "	(120)
Horse	-	1,500	"	" "	(78)
Cat	-	2,000	"	" "	(78).

Redenbaugh et al. (128) have determined the insulin content of the chicken pancreas. The pancreases from one hundred chickens (330 gm.) were removed and insulin prepared by Fisher's modification of the Doisy-Shaffer method. They obtained a yield of 7600rabbit units per kilo of pancreas and concluded that the chikken pancreas, by weight, contains as much insulin as calves' pancreas. Relatively large amounts of insulin were also found in the kidney and kiver.

The fish pancreas as a source of insulin has been studied extensively. As early as 1889 Diamare recognised that certain structures in the abdominal cavity of some of the Teleostii are identical with those of the islands of Langerhans of higher vertebrates. In 1903 Rennie noted in each species of Teleostii at least one large islet, which is often encapsulated, and thereby separated from the zymogenous tissue. Jackson (123) and McCormick (124) undertook a thorough investigation of the islets in fishes. In order to determine whether the islets of Langerhans or the zymogenous tissue are the real source of insulin in the pancreas; Macleod (125) prepared alcoholic extracts from the principal islets of a number of bony fishes, including the sculpin and angler fish. Notwithstanding the poor yields he obtained, due to the method of extraction, he showed that very potent extracts could be prepared from the principal islets. He consequently suggested that the islets might furnish a convenient practical source of insulin, which aspect was subsequently investigated by

/McCormick

McCormick and Noble (18) in America, and Dudley (79) in England. The former workers, employing an acid alcohol method of extraction, obtained large yields of insulin at relatively small cost from large quantities of fish. From the cod (Gadus callarius) they obtained average yields of 6,000 rabbit units per kilo of principal islets, and a similar yield from the halibut (Hippoglossus hippoglossus). From pollack and haddock yields as high as 12,000 and 9,000 rabbit units respectively were obtained in isolated cases. Dudley (loc.cit), making use ofaqueous picric acid for fixation, obtained 13,120 rabbit units per kilo from the cod and concluded that "the collection of fish islets by the above method will be found to have advantages wherever the circumstances make this source of insulin worth exploitation." (loc.cit.). Similar investigations were conducted by Vincent, Dodds and Dickens (126) on Lophius piscatorius and Myoxocephalus bubalis, who confirmed the earlier work of Macleod and showed that the islet tissue yields 6 to 7 times as much insulin as the zymogenous tissue. Their results were in general agreement with those of McCormick and Noble and Dudley. Jensen et al. (129), making use of acid alcohol extraction, prepared crystalline fish insulin (using Abel's method) from the islet tissue of the cod and pollock. Unfortunately, they did not report on the yields per weight that they obtained.

Practically all commercial insulin is at present prepared from the pancreas of either beeves or pigs. Recently (87) fish pancreas has been put to use for this purpose in Norway. Islets from the cod, saithe and halibut are now being collected on the trawlers and preserved for working up ashore by the acid alcohol and picrate method. It has been found that the insulin content is much higher (often 10 to 20 times) than in

/mammalian

mammalian pancreas, but the disadvantage is that the individual organs in the fish are disproportionately small.

In 1924 Fenger and Wilson (120) supplied the following information: "The number of domestic animals slaughtered yearly in the United States under federal government inspection approximates 8½ million cattle, 4 million calves, 11 million sheep and lambs and 43 million hogs. These animals furnish about 5½ million kilos of fresh pancreas glands." They concluded that "the available supply of raw material is, therefore, far in excess of any possible demand for insulin."

Since then the position as regards supply and demand has changed considerably for reasons to be furnished presently. In 1947 the Interim Commission of the World Health Organisation (131) sent a questionnaire to governments asking for information on their consumption and production of insulin, and for an estimate of their prospects in regard to insulin supplies for the next ten years. By April, 1948, replies had been received from 44 countries and the following figures give only a broad indication of insulin needs and production:-

Excluding the United States and Canada, for which figures were not available, the annual consumption of insulin amounted to 3,901,000,000 international units. It has been estimated that insulin comsumption for the following ten years, with the exception of those countries already mentioned, will total approximately 46,425,000,000 units. This, however, does not include the requirements of the United States, which has one million diabetics and which can export only a relatively small quantity of insulin (loc.cit.).

The present yearly production of insulin in 43 countries is 11,087,300,000 units, of which most is produced by the United States, followed by the United Kingdom

/and

Kingdom and the Netherlands. It is estimated that the total production for the next ten years may reach 115,250,000,000 units.

Of the 44 countries that had replied to the questionnaire, 34 were not self-sufficient as regards their insulin supplies. Their import requirements have been estimated at 1,854,000,000, while the exportable surplus of the producing countries is only 1,430,000,000 units, which shows a yearly deficit of 424,000,000 units (loc. cit.). In order to balance this, many countries have taken steps to increase production: obligatory collection of pancreas from slaughtered animals, granting of subsidies to encourage collection, installation of suitable refrigeration apparatus at slaughterhouses, training of factory personnel for this purpose, etc. It is to be hoped that, partly through these measures, and partly by the increased yield of insulin expected as a result of improved methods of extraction, the exportable supply will meet the demand. Nevertheless, a shortage of insulin at some future stage is not beyond the realms of possibility.

Probably the chief causative factor of this state of affairs is the disproportionate increase of production and consumption. Production is firstly hampered by present currency restrictions; secondly, much raw material goes waste in the non-producing countries; thirdly, the world's cattle stock has declined considerably during the war and post-war years. Consumption has increased through more efficient methods of diagnosis of diabetes, increasing numbers of surviving diabetics and chiefly as a result of the development of well-organised medical services and skilled medical profession in hitherto less developed countries.

/In

In face of a threatening shortage, it has been thought advisable to investigate some potential sources of insulin supply which could be utilised should the necessity arise. An additional consideration has been the theoretical interest attached to the determination of the insulin content of the pancreas in hitherto unexplored sources. For these reasons the insulin yield from the following sources has been determined in this investigation:-

(i) whale, (ii) shark, (iii) South African domestic animals - cattle, pigs and sheep.

/(1) WHALE INSULIN.

(1) WHALE INSULIN.

That the whale pancreas could be an important potential source of insulin, is indicated by the following figures representing the total antarctic catch, including land stations, for the years 1947-1950 (132).

TABLE 31.

Total antarctic catch including land stations.

Season	Blue	Fin	Humpback	Sei	Sperm	Total.
1947/1948	6908	21141	26	621	2622	31318
1948/1949	7625	19124	31	577	3903	31260
1949/1950	6182	20060	2143	1284	2727	32396

If the average weight of the whale pancreas is considered as 120 pounds and, if it were assumed that whale pancreas would yield per weight the same unitage of insulin as beef pancreas (i.e., 2,000 units per kilo), then the total amount of insulin to be derived from this source would amount to 3,456,000,000 units annually. This amount almost equals the annual consumption, excluding the United States, and is more than double the import requirements of 34 countries (see preceding paragraphs). The impressiveness of these figures prompted the present investigation. However, before discussing the directly relevant aspects, it is felt that, for a proper appreciation of later statements, certain information regarding whales in general and whaling methods should be furnished.

/Suborder

Beddard (202) gives the following classification of whales: (not complete).

ORDER: CETACEA.

Suborder	Families	Genera	Species.
Mystatoceti (Baleen whales)	Balaenidae	Balaena (right whales)	Balaena mysteticus (Greenland whale)
			Balaena australis (Southern right whale).
			Balaena glacialis
		Neobalaena	
	Balaenopteridae	Balaenoptera (rorquals)	Balaenoptera sibbaldius
			Balaenoptera musculus (blue whale)
			Balaenoptera physalus (fin whale)
			Balaenoptera borealis (Sei whale)
			Balaenoptera rostrata
		Megaptera (humpback)	Megaptera nodosa (humpback whale)
	Rhachianectidae		
Odontoceti (toothed whales)	Physeteridae (sperm whales)	Kogia (pigmy sperm whales)	
		Physeter	Physeter catodon (sperm whale)
	Ziphiidae (beaked whales)	Mesoplodon	
		Hyperoodon	
		Ziphius	
	Delphinidae (Dolphins & porpoises).		

Vosmaer (203) describes mystatoceti as: possession of whalebone, therefore no teeth, characters more like mammals, two nostrils; and odontoceti as: No whalebone but teeth, one nostril, cranium asymmetrical. Regarding odontoceti, Beddard writes: "It contrasts markedly with mystatoceti, the differences being so great that more than one naturalist is disposed to give to the two a different line of descent."

Whalebone whales feed exclusively on plankton, including fishes. They obtain food by means of a whalebone sieve and as much as two tons of plankton have been taken from the stomach of a blue whale (215). Sperm whales are mainly dependent on cuttle fish, some of which must be obtained at considerable depths (loc.cit.).

The blubber in the sperm whale may be as thick as 14 inches and up to 20 inches in the balaenidae. In the sperm 4 tons of blubber yield 3 tons of oil; in addition to this the sperm contains a large amount of clear oil in the head. To preserve this, sperm whales are invariably shot in the body.

The most frequently hunted whales in the antarctic are the Fin (Balaenoptera physalus), Blue (Balaenoptera musculus), Sei (Balaenoptera borealis) Humpback (Megaptera nodosa) and Sperm (Physeter catodon). From the classification it is noted that the first three belong to the same genus and with the Humpback to the same family, viz: Balaenopteridae. The sperm, on the other hand, belongs to the suborder odontoceti. With a view to later findings, it is of interest to bear this relationship in mind.

Having arrived in the whaling grounds, the catcher vessels (± 300 tons) operate at varying radius from the factory ship, to which dead whales are brought in daily. The harpoon weighs 75 kilos and it carries a war head of cast iron, weighing 9 kilos, which is filled with black

/powder.

powder. It is supplied with a time fuse (3 to 4 seconds) causing explosion of the powder as soon as the weapon has struck its object. The harpoon is fired off by 210 gm. powder consisting chiefly of nitro-cellulose (133).

The animals are shot at an average distance of 20 to 30 fathoms. They appear above the water, head first, describing a semicircle and are seen above the surface for no longer than 3 to 4 seconds, during which short space of time the harpoon is fired. There is no time for accurate aiming; frequently a harpoon misses its goal exploding in the water. Where a hit is registered, it invariably strikes the body; the harpoon penetrates an average distance of 4 feet (in most cases into the abdominal organs) before the charge goes off, causing a cavity of about 18 inches diameter. If the first harpoon does not kill, the animal is pulled in with the attached harpoon rope and loose, less charged harpoons fired at short range. As a rule, 1 to 3 of these are necessary in order to kill the animal. Depending on the site where it has been struck, it may die immediately or an hour or more may elapse before it finally ceases its struggles. However, ½ hour can be considered a good average time for killing the animal (loc. cit.). As the explosive head of the harpoon most frequently bursts in the intestines, it spreads putrefactive organisms throughout the circulation during the time taken for the creature to die. Only in the comparatively few cases when the whale is speedily and humanely killed by a harpoon striking a vital spot altogether away from the intestines can satisfactory meat be produced from it (134).

The dead whale is inflated with compressed air, 5,000-6,000 litres being used per animal, and left thus floating on the water, belly upwards. In this condition they are left for periods varying from ½ to 18 hours,

/with

with an average of 8 to 12 hours, before being towed to the factory ship. Here they are speedily worked up at the rate of 1 Blue whale per hour, 2 Fin whales per hour and 3½ Humpbacks (133).

During the past season the more humane method of killing by electrocution was experimented with. The harpoon is of the same weight; to it is attached a highly charged, insulated electric cable. However, as this cable is much lighter than the conventional harpoon rope, it snaps, the dead whale sinks and is lost. On account of this, the new method has, as yet, not met with much success (loc. cit.).

The advantages of whale pancreas as a possible source of insulin are many. The size of the organ is such that it is accessible and easily handled (170 - 200 pounds in the Blue whale, 110 - 120 pounds in the Fin and 50 - 60 pounds in the Sperm) (loc. cit.). That this is of great importance as far as commercial production is concerned, is evidenced by the fact that, although fish pancreas is often 10 to 20 times richer in insulin than beef, it is not used for this purpose to any appreciable extent. The reasons for this is that the comparatively small organs in the fish require more time, skill and labour to remove, which, in its turn, increases production costs. According to the W H O report already referred to (131), production cost for fish insulin may exceed that for beef insulin many times. Secondly, the large number of whales killed annually could, under optimal conditions, furnish a large portion of the world's insulin demand and thus contribute much to ward off an insulin shortage. The figures presented in Table 31 and succeeding paragraphs fully bear this claim out. Thirdly, the whale pancreas at the moment is waste material; its collection on board the factory ship will not involve much extra

/cost

cost, so that it can be disposed of at comparatively low price.

Major disadvantages of this potential source upon which attention is immediately focussed are the following: The method of killing is such as to enhance the action of putrefactive organisms. Added to this is the high temperature which is maintained within the body of the dead animal. The thick layer of blubber insulates it so effectively that, for as long as 24 hours after death, the inner temperature remains almost constant, notwithstanding the iciness of the water in which it floats (133). Thirdly, the long periods that the animals are left floating would, in combination with the previously mentioned two factors, certainly affect the insulin content adversely.

This field of research is practically "virgin soil".

In all the available literature only one reference, as regards whale insulin, was found (135). Jacobsen (loc.cit.) collected ovaries, testes, thyroid, adrenals and pancreas from the Blue whale and subsequently extracted these with a view to determining their hormonal yields. In many respects the present investigation, therefore, seems to have been the first of its kind (as far as can be judged from current published work).

Points of major interest which presented themselves and were dealt with in this investigation are the following:-

(i) The insulin content of the whale pancreas expressed as international units per weight of material;

(ii) Species variations in insulin content, if such exist;

(iii) Sex variations in insulin content, if such exist;

/(iv)

(iv) The effect of post mortem time and related factors on the insulin content.

(1) Material and Methods.

The material for this investigation was kindly supplied by Messrs United Whaling Ltd. of London. It was collected in the Antarctic during the 194891949 whaling season and delivered at CapeTown on the return journey of the whaling fleet. Altogether 38 samples were received,representing 12 Blue whales, 17 Fin, 6 Sperm and 3 Humpback whales, the post mortem time for these varying from 2 to 10 hours. Samples were received during March, 1949, and extraction continued during the subsequent months. Accompanying each sample was the necessary information as regards the species of whale, date of killing, size, sex and exact post mortem period, i.e., the lapse of time from the firing of the first harpoon until the whale was brought aboard, the pancreas removed and treated.

All samples had received the following treatment: The pancreas was removed and the entire organ finely minced; the requisite amount of picric acid being added during the mincing process. The reasons for reverting to the picric acid method were twofold:- Firstly, the distribution of islet tissue in the whale pancreas (i.e., head, body and tail) has as yet not been studied. The organ is too large to extract the entire bulk in a series of samples; removing only part of it might involve the risk of obtaining more or less densely islet populated areas, resulting in a possible misrepresentation of facts. The picric acid method allowed the mincing of the whole organ, thorough mixing of the picrated mass and subsequent removal of a specimen sample,

/extraction

extraction of which would yield insulin representative of the whole organ. Secondly, the method was, at the time, being investigated and had been shown to work efficiently, easily and rapidly with small quantities of material. In view of the extremely high oil content of the pancreas, it was feared that an alternative method might have proved difficult or unsuccessful.

From the picrated mass of each pancreas 1.5 - 2 kilos of material were removed. This was immediately deep frozen into slabs of about 12 x 8 x 1½ inches and kept in cold storage for the remainder of the whaling season and return journey to Capetown. On arrival here, the slabs were transferred and kept in cold storage by Messrs Imperial Cold Storage Ltd. From this central depot the frozen slabs were removed singly as required and extracted. On account of the number of samples, it is clear that some samples had been left in cold storage for periods of six months or longer.

Extraction was done according to the method described in Part II of this work. However, as whale insulin was still largely an unknown entity, it was decided to subdivide the material of each slab into smaller samples of 200 gm. each. These were then extracted under varying experimental conditions, the results of this having been partly quoted in Table 28, page 116. . Further results of this nature are not relative to the subject under investigation, so that those given in the following Table 32 represent only the maximum yield of insulin obtained from the specific original sample.

The final product obtained in each instance was the crude insulin hydrochloride; none of the yields having been purified beyond this stage. The hydrochloride was tested for activity as soon as possible after its

/preparation

preparation according to the method described in Part I. Altogether 87, 200 gm. samples were extracted, these having been tested in 115 assays. In order to check these, 10 full assays according to the mouse method were carried out.

At the end of the 1949/1950 season 5 further samples of pancreas were obtained from the same source. These were much larger than the previous ones (20 - 25 kilos each) were frozen only (with no picric acid added) and were to be used for alcohol extraction and preparation of crystalline insulin. The method followed was that of Romans, Scott and Fisher (77).

(2) Results:

TABLE 32.

Yields of Insulin from whale pancreas.

Serial Number	Species	Sex	Post mortem time (hours)	Yield insulin hydrochloride gm/200 gm. sample	Activity crude hydrochloride I.U/mg.	Insulin Content IU per kg. of pancreas
1	Sperm	Male	3½	0.488	0.62	1510
5	"	"	3	0.572	0.84	2002
6	"	"	3	0.634	1.12	3540
8	"	"	2	0.536	(1.16 (1.10	(3108 (2948+
9	"	"	9½	0.617	(0.98 (0.90	(3023 (2776+
10	"	"	8½	0.372	0.40	744
11	Fin	Male	2	0.514	(0.42 (0.48	(1080 (1234+
13	"	"	3	0.362	0.46	832
18	"	Female	6½	0.487	0.22	535
19	"	Male	4	0.463	0.14	324
21	"	Female	2½	0.360	0.29	522

/22.

Table 32 (continued).

Serial Number	Species	Sex	Post mortem time (hours)	Yield insulin hydrochloride gm/200 gm. sample	Activity crude hydrochloride I.U/mg.	Insulin Content I.U per kg. of pancreas.
22	Fin.	Male	7½	0.280	0.19	266
29	"	"	8	0.435	(0.48 (0.44	(1144 (958†
32	"	Female	5	0.327	0.16	261
33	"	"	6	0.202	0.04	44
34	"	Male	2¼	0.252	0.06	75
36	"	"	2¾	0.860	0.12	515
37	"	"	9½	0.244	(0.36 (0.40	(440 (488†
38	"	Female	7½	0.412	0.10	206
39	"	"	8	0.709	0.22	780
40	"	Male	4½	0.342	0.11	186
41	"	"	6	0.356	0.21	374
44	"	Female	3¾	0.390	0.29	565
45	"	Male	4½	0.354	0.31	548
12	Blue	Female	2	0.653	(0.48 (0.52	(1518 (1698†
15	"	Male	8½	0.710	0.44	1562
16	"	Female	3½	0.864	0.32	1330
23	"	Male	4	0. 420	0.35	735
24	"	"	4¼	0.522	(0.42 (0.44	(1096 (1148†
25	"	"	6¼	0.458	0.23	526
26	"	Female	6	0.540	0.23	620
27	"	"	3¾	0.640	0.17	544
28	"	Male	5	0.643	(0.26 (0.30	(835 (964†
30	"	"	3½	1.231	(0.29 (0.26	(1785 (1600†
31	"	Female	2½	0.574	0.18	516
35	"	Male	2½	0.792	0.13	514
46	Hump back	Male	4¼	0.468	0.34	795
47	"	Female	4	0.384	(0.62 (0.65	(1190 (1248†
48	"	"	3	0.314	0.65	1020

† as determined by mouse assay.

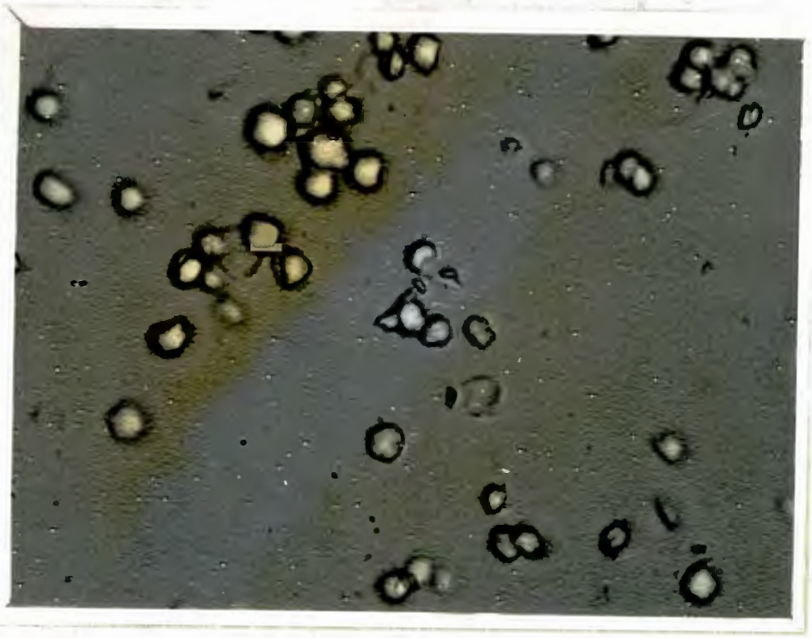
Two unsuccessful attempts were made to prepare crystalline insulin from whale pancreas. The extremely high oil content of the organ made the initial extraction with acid alcohol and subsequent distillation very difficult. The material obtained after the first salting out was rather gelatinous and not altogether soluble in acidulated water. Nevertheless, a copious iso-electric precipitate was obtained but attempts to crystallise this material were not successful.

In a third attempt 20 kilos of Blue whalepancreas were used. The animal had a post mortem time of 5 hours and a small sample of material extracted by the picric acid-acetone method, had indicated an insulin content of 1,280 units per kilo. During the mincing of the material small amounts of 3N hydrochloric were added, the minced mass well stirred and as much oil as possible separated, by gentle pressure through a layer of cheese cloth. The residue was added to the alcohol mixture as soon as possible. Distillation of the alcohol was done quickly with slightly raised temperature. An abundant iso-electric precipitate was obtained, but the final product appeared amorphous. After twice repeating the process of redissolving the final product in water plus acetic acid, carrying the process through the different steps of the method (77), a small yield of very tiny crystals was obtained (shown in Figure 11).

The yield from 20 kilos was 0.277 gm.and the activity, assayed by the mouse method, 19.8 units per milligram (i.e, 275 units per kilo).

/Figure 7.

Figure 7. Crystalline Whale Insulin. x 800.

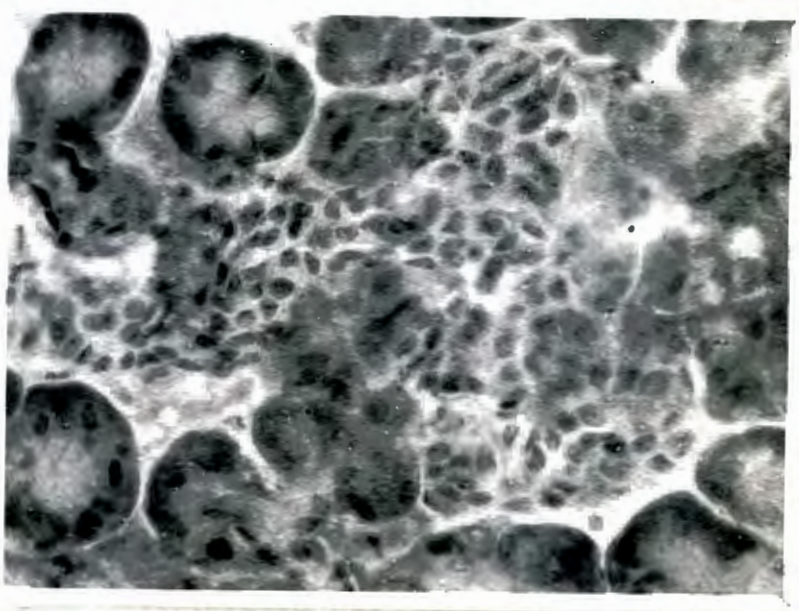


Comparison with the photomicrograph of beef crystalline insulin (Fig. 19) shows the crystals to be of the rhombohedral type and of approximately the same size.

3. Discussion.

The photomicrograph in Figure 8 shows the histological structure of the Fin whale pancreas. The piece of tissue was removed at random from the frozen material used for alcohol extraction and it is, therefore, impossible to state what part of the organ it represents.

Figure 8. Histological structure of Fin whale pancreas. x 450.



As a detailed study of the histological structure of the pancreas was not included in this investigation, no further sections were prepared from any of the other species. Neither was a differentiation of the different types of islet cells undertaken. Nevertheless, from the above and similar sections it was established that, generally, and with regard to its islet tissue, the whale pancreas does not seem to differ from that of other mammals. In the different sections studied, the absence of islets from large areas, or their very scanty distribution, was especially marked. Whether this is characteristic of the entire organ is impossible to say.

The overall impression gained from the results shown in Table 32 is that the whale pancreas is, generally speaking, poor in insulin. (This statement refers to the pancreas as obtainable under optimum conditions of whaling procedure, i.e., immediate killing of the animal, speedy removal to the factory ship and treatment of the pancreas for extraction purposes, which, under most favourable conditions, cannot be achieved in less than 2 hours). Excepting the sperm pancreas, the maximum yieldsobtained from the other three species vary from 1200 - 1800 units per kg. of material (this representing the activity contained in the crude hydrochloride as prepared by the picric acid-acetone method). As compared to the amount of insulin contained in beef and pig pancreas, which, by this method of extraction, had been shown to be of the order of 3000 - 4000 units per kilo, it can be stated that whale pancreas has per weight only $\frac{1}{2}$ - $\frac{1}{3}$ of this value. It, therefore, shows closer correspondence with the sheep pancreas, which has been shown to contain appreciably less insulin than either the pig or beef (120). No explanation can be offered for this low insulin content of the whale pancreas.

/An

An interesting observation is the very marked species difference shown by the Fin, Blue and Humpback on the one hand, and the Sperm on the other. Even the former three show slight differences among themselves. The Fin pancreas contains the least insulin and the maximum yield obtained from it was 1,000 - 1,200 units per kilo. Altogether 18 samples of Fin pancreas were extracted and the possibility that none of the results reflect the actual position, although slight, cannot be altogether ignored. A possible masking effect of factors such as particularly quick destruction of insulin in the Fin is discussed in a later paragraph. That this should be so is unfortunate, because these whales are killed in largest numbers (20,000 during the 1949/1950 season - Table 31), and their pancreas is of considerable size - 110 - 120 pounds. Apart from other factors to be presently considered, the Fin pancreas, notwithstanding the large amount available, could not assume much importance as a potential source of insulin.

Of the Humpbacks only 3 samples were extracted. Whether the values obtained represent the maximal ones, is difficult to judge, as several factors could have been responsible for a decrease of insulin content. However, taking the results at face value, it would seem as if the insulin content (1,000 - 1,200 units per kilo) in this species closely approximates that of the Blue, (see later). Humpbacks have for several years been protected (c.f. Table 31), but, apart from this, are killed in comparatively smaller numbers.

The Blue whale pancreas is especially large (170-200 pounds) and, with 12 samples that were extracted, a maximum yield of 1,500-1,700 units per kilo was obtained. This figure is slightly higher than that reported by Jacobsen (135) who had found 1,000 units per kilo for the Blue

/whale,

whale, but could probably be accounted for by the different method of extraction without further purification, by different degrees of freshness of the material or by individual variations.

Standing in sharp contrast to the above is the high insulin content of the Sperm pancreas. In six samples that were extracted a maximum yield of ±3,000 units per kilo was obtained, which is only little less than that extracted from beef. This difference is not so surprising if it is borne in mind that the sperm is a distinct suborder, differing in many respects from the other species. Again, this is, unfortunately, so, because not only is the sperm pancreas of smaller size (50 - 60 pounds) but these animals are killed in muhh lesser numbers.

From the quoted results it is impossible to distinguish any difference in insulin content that could be ascribed to sexual variations, both males and females having given good yields. It is interesting to note, though that the sperms were all males - the Antarctic has no sperm females because these inhabit the warmer waters. It is believed that the old sperm whales leave the herd and migrate to the Antarctic to die there, thence their description by whalermen as "the old gentlemen of the Antarctic". No samples of female Sperm pancreas were supplied and a comparison is, therefore, impossible. In view of the fact that the sperms were all old animals and that the insulin content of the pancreas decreases with age (c.f.work of Fisher and Scott (121), on the insulin content in cattle of various ages), it could be assumed that in younger animals the amount of insulin would be appreciably more.

/Viewing

Viewing the results of Table 32, a striking feature is the variations in yield shown by samples from the same source. From the Fin variations from 50 - 1,100 units per kilo were encountered; Blue 500-1,800 and Sperm 700-3,000. Simultaneously with this are noticed the variations in post mortem period, extending from 2 - 10 hours. This leads to a consideration of the causative factors - is it the post mortem period per se which is responsible for these variations or other identified and unidentified factors ?

Regarding the post mortem period, it will be noticed that there is nothing indicating a graded decrease in insulin content with increasing time. If this were the case, it would be expected that, in general, those samples with the shorter post mortem periods, would be the ones giving the higher yields. That this is not the case is clear. On the contrary, some of the highest yields obtained were from samples with long post mortem periods (c.f.sample 9 - 9½ hours - 2,800 units, sample 29 - 8 hours - 1,000 units, sample 28 - 5 hours - 1,000 units. On the other hand, samples with shorter post mortem periods frequently gave extremely low yields (c.f.sample 34 - 2¼ hours - 75 units, sample 19 - 4 hours - 324 units, sample 31 - 2½ hours - 516 units, sample 35 - 2½ hours - 514 units. It appears, therefore, that the post mortem period as such is not the chief factor in causing insulin destruction. As criterion for determining the amount of insulin destruction, has been taken the maximum yields obtained from the particular source. That it undoubtedly has an adverse effect will not be denied, but it seems more probable that the disappearance of insulin from the pancreas, due to a time factor chiefly, is a much more gradual process. Other factors excluded, it seems as if leaving the dead whales floating until towed

/to

to the factory ship could, within limits (maximum period tested - 10 hours, c.f. sample 9), not result in great destruction of insulin.

An accompanying phenomenon which is frequently considered causative of insulin destruction is temperature; thence the generally practised precaution of deep freezing material to be used for extraction. The effect of increased temperature is chiefly that of enhancing putrefactive processes in the dead organism. Again, the same argument can be used that high temperature (which is maintained in the dead whale) alone or temperature plus post mortem period cannot be responsible for the degree of insulin destruction observed in this series of extractions.

It was impossible to obtain information as regards the number of harpoons used for each animal, or the exact spot where the different whales were struck. It seems, however, that the varying degree of insulin destruction is closely related to the site of explosion of the harpoon head. In those samples, giving high yields of insulin (e.g., Nos.6, 8, 9, 11, 12, 22, 24 and 30) the whales had probably been struck in some vital spot such as the brain or heart, so that they had died almost instantaneously. It is wellknown that, with a head or thoracic wound, putrefaction is less likely to start and spread than with an abdominal wound. This would then explain why some samples, notwithstanding a long post mortem time, still yielded amounts of insulin equal to those obtained from the freshest possible samples. On the other hand, the extremely low yields would probably be the result of abdominal wounds, where the intestines and related organs had been destroyed. Damage to the intestines results in the quick spread and action of putrefactive organisms. Lillie (134) states

/that

that this occurs almost immediately and that the responsible organisms are spread via the bloodstream during the time the animal takes to die. The low yields of samples 25, 34, 36, 38, 40 must then be ascribed to destruction of the insulin through putrefaction. This process is obviously enhanced by the high internal temperature of the dead whale. Putrefaction, caused by the method of killing the whales, is probably the greatest single factor in the destruction of the hormone. In one case sections of the pancreas (Blue whale, post mortem 7½ hours) revealed the tissue to have autolysed beyond recognition.

It was pointed out that the Blue whale pancreas contains slightly more insulin than the Fin (as determined from maximal values obtained). It is interesting to compare the yields obtained as affected by post mortem time and/or putrefaction. In the Fin 6 of the 18 samples had yielded virtually no insulin (less than 300 units per kilo) whereas in the Blue not a single sample (from 12) had yielded less than 500 units. The average post mortem time in the former was 4.8 hours and in the latter 4.3 hours. This suggests that either the insulin content in the Blue whale is, in the intact organ, much higher than was revealed by any of the samples, or the Fin harbours in its intestines larger masses of those organisms causing putrefaction. It appears probable that the latter or some related factor is the case, rather than the former. If, in only 6 samples of Sperm pancreas (average post mortem period 4.8 hours) as many as 50 per cent had shown maximum yields, it could be expected that in 12 samples of Blue whale at least one or two yields would be representative of the insulin content. Not only is the activity of the hydrochloride from the Fin less than that from the Blue (average 0.24 units per mg. as compared

/to

to 0.30), but also the actual amount of hydrochloride is less (average 0.408 gm.as compared to 0.596 gm.) This seems to indicate that, not only does the Fin contain less insulin, but also its destruction is more rapid. (The varying activity of the hydrochloride obtained from different sources has already been commented on in Part II. of this work). The highest activity of the hydrochloride obtained for Fin is 0.48 unit per mg, for Blue 0.52, for Humpback 0.65 and for Sperm 1.16. This latter figure approximates the average activity obtained for beef hydrochloride. Judged from these results, it appears that whale pancreas contains amounts of insulin in the following decreasing order:-

(i) Sperm approximating beef pancreas,
(ii) Humpback " ½ " "
(iii) Blue " 1/3-½" "
(iv) Fin " 1/3 " "

It is interesting to note that the insulin content, as indicated here, reflects fairly accurately the classification of whales. The sperm, with its high yield, belongs to a different suborder, viz: odontoceti. It would be interesting to determine whether the pancreas in the other families of this suborder (i.e., beaked whales, dolphins and porpoises) contains similar relatively large amounts. On the other hand, the mystatoceti are poor in insulin. The Blue and the Fin belong to the same genus, balaenoptera, and yield approximately the same amounts. Based on this assumption, the Sei (from which no samples were received) would probably show an equally low insulin content. In the Humpback, belonging to the genus megaptera, the insulin content could be slightly higher than the values found from three samples only.

The question arises whether the results obtained

/(maximum)

(maximum) represent the true insulin content of the whale pancreas. Under even the most favourable conditions of whaling, it very rarely happens that a sample fresher than two hours post mortem is obtainable and the removal of a perfectly fresh pancreas (i.e., from 10 - 20 minutes after the animal had died) is virtually impossible. As all the extracted samples were of 2 hours or more post mortem, the possibility is not excluded that considerable insulin loss had occurred during that period, so that the actual insulin content is much higher than that reflected by the results. Granted that such a loss does occur, it would be expected that this process would continue at increased rate, arriving at a stage, sooner or later, where the insulin content is nil. How a 9-hour sample under such conditions could still give a 3,000 unit yield would be difficult to explain. It appears more feasible to assume that the rate of insulin destruction during the first two hours is not of such a degree as to greatly affect the insulin content (except where extensive putrefaction is the causative factor).

It could be argued that insulin loss had occurred due to the method of preservation of the material over a period of several months. That freezing preserves the hormone for at least six months has been amply confirmed; that picric acid likewise preserves it has been shown; that the two methods combined would have any other effects than these is highly improbable. The first samples were extracted two weeks after collection and the last ones 6 months. If there had been a progressive loss during this time this would have been reflected by decreasing yields. This was not observed.

The picric acid-acetone method has been used for insulin extraction from sources as varied as mammals,

/fish

fish and birds. It is highly improbable that this method, with all the modifications tried out, would fail to extract all the insulin from the whale pancreas.

4. Conclusions.

The pancreas in the Fin whale, Blue and Humpback, is naturally poor in insulin, whereas that of the Sperm approximates beef pancreas. It can, therefore, not be considered a promising source for the commercial extraction of insulin. Added to this is the extensive insulin destruction that occurs in the organ prior to its removal and processing. The antiquated method of whaling is solely responsible for this. Putrefaction, caused by the explosion of the harpoon head in the animal body, and enhanced by the high temperature, is the greatest single factor causing insulin loss. More humane methods of killing, as electrocution, would result in less damage to the tissues and larger yields of insulin, as the post mortem period and temperature alone do not involve considerable loss. Under prevailing conditions, however, there is no prospect of whale insulin contributing to meet the increasing world demand for insulin.

(2) SHARK INSULIN.

(1) Introduction.

The histological structure of the mammalian pancreas and the cytology of the islet cells were extensively studied by Laguesse, 1906-1911 (204), Rennie, 1909 (205), Lane, 1907 (206), Bensley, 1911 (207), Otani, 1929 (208), and Thomas, 1938 (209). Through these and other studies it was established that the islets may be located (i) in the interlobular connective tissue, but connected with the duct system by solid cords; (ii) in the lobules, unconnected with the acini but connected with the intralobular duct system; (iii) in the lobules and in connection with either acini or ducts (these include the great majority); (iv) in the interstitial tissue or intralobularly, but not connected with either acini or ducts. Due to the character and chemical properties of their cytoplasmic granules, three granular cell types have been distinguished, viz:, A, B, and D. In the dog Hunt found these to constitute 20, 75 and 5 per cent respectively. Whether the D-cells are a separate type or whether they represent a stage in the development of the A- or B-cells, remains to be determined. In addition to these three, Bensley has described a fourth cell type in the guinea pig pancreas - the non-granular C-cell, which has been suggested represents the progenitor of the A-cells. The use of alloxan has definitely established the B-cells as responsible for insulin secretion.

Regarding the A-cells, the possibility has been suggested that they may be concerned with the production of the hyperglycaemic-glycogenolytic (H-G) factor (210), the presence of which has been revealed in the pancreas of all species tested (211). As a result of the readiness with which A-cells stain with silver (212), they have been regarded as related to endodermal argentophil cells in the

/gastrointestinal

gastrointestinal tract, where Tohver (213) noted relatively large numbers present in the upper two-thirds of the mucosa of dog stomach, from which considerable amounts of the H-G factor have already been extracted (214).

Reference has already been made (page 148) to the studies of McCormick and Noble, Macleod, Dudley, Dodds and Dickens, etc. on the extraction of insulin from fish pancreas. In the Teleostii it varies widely structurally and topographically. Either it lies in the mesentery, when it is divided into three parts (cranial, intermedial and caudal mesenteric) (216), or in the liver forming the so-called intrahepatic pancreas, or it may occur next to the portal veins. Apart from this, there occur in bony fishes certain glandular structures found by Remmie (1903) to be homologous to the mammalian islets of Langerhans, and the largest of them termed "principal islets" by him. The extraction of large amounts of insulin from the isolated and generally encapsulated, principal islets and of smaller amounts of insulin from the pancreatic zymogenous tissue has already been referred to (page 149).

In the Elasmobranchii the pancreas is large and compact and situated near the duodenum as a dorsal and a ventral portion. The islands are large and numerous and retain their primitive relationship to the ducts. Although they show a marked resemblance to the higher vertebrates as far as shape and distribution are concerned, they represent the most primitive type of islet structure in vertebrates (217).

A well-known elasmobranch species of the Cape waters is the "vaalhaai" or Galeorhinus capensis. During the war years, and immediately afterwards, these sharks were caught in large numbers for the extraction of

/vitamin

vitamin A oil from the liver. As many as 13,000 of these fish were caught per month by a single concern (218). It was, therefore, thought fit to investigate the structure and insulin content of the pancreas in this species. The study of the histological structure of the pancreas was undertaken in collaboration with D. R. de Villiers, formerly of this department, and made the subject of a thesis by the latter author (219). The results will, therefore, not be recapitulated in detail, but only brief reference made to them. Since these animals were caught in such large numbers (these have lately been reduced considerably due to the synthesis of Vit.A.), since their pancreas is a fairly large, compact organ (30 - 60 gm., showing seasonal variations in weight (220) and since the fish pancreas generally has been shown to give good yields of insulin, the extraction of the hormone from this source was fully investigated.

(2) Material and Methods.

In order to obtain the material sufficiently fresh for microscopic examination and insulin extraction, the writer, on several occasions, accompanied the trawlers of Messrs Marine Products, Cape Town, on their shark fishing trips. The fish were caught by handline and weighed from 40-±70 pounds. Once on board, the abdominal wall was opened and, on spreading out the viscera, the pancreas could be seen as a distinct, solid, yellowish organ adjacent to the alimentary canal at the junction of stomach and duodenum. Small portions were removed from different parts of the organ and these dropped into the specific fixatives, seven of these containing mercuric chloride and six bichromate as potassium bichromate in solution, having been used.

/As

As the trawlers were not fitted with freezing apparatus and, on occasions, stayed at sea for five to six days, the picric acid-acetone method was reverted to for the extraction of insulin. The pancreases were removed immediately the fish were brought aboard and cut up with scissors into specimen jars containing a saturated aqueous solution of picric acid. In one instance the material was minced, with picric acid crystals added to it, during the process. Extractions were made as soon as possible after return to the laboratory; in no case did the period of fixation exceed eight days.

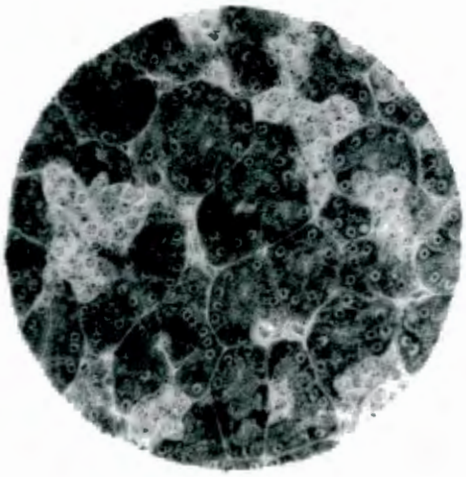
The method of extraction was that outlined in Part II of this work. In each instance the crude hydrochloride was prepared, no attempts having been made at further purification. Neither was the hormone prepared in its crystalline form. The hydrochloride was tested for activity by the method described in Part I of this work; in addition one sample was tested by the twin cross-over rabbit method.

(3) Results and Discussion.

Regarding its microscopic appearance, the observations of earlier investigators were confirmed. The striking "helle Felder" of Oppel are shown in Figure 9. These light fields, irregular in shape, size and distribution, are sharply demarcated from the surrounding zymogenous tissue. With specific staining methods it is seen that they consist of islet cells, chiefly arranged around the epithelium of the smaller ducts.

/Fig.9.

Figure 9.



X 38.

Fixative: Zenker-formol.

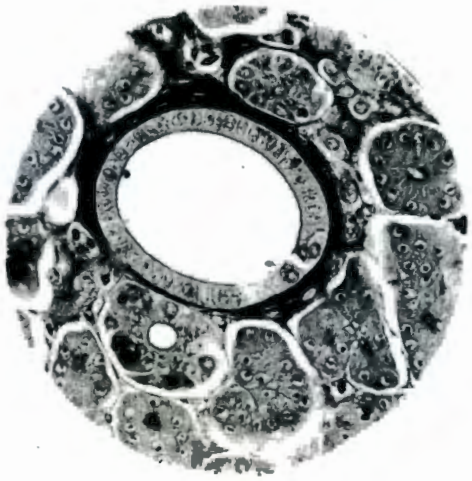
Stain: Pyronin methyl-green.

The primitive relationship of the islet cells to the duct epithelium is well-illustrated in the succeeding sections. As shown in Figure 10, groups of islet cells occur alongside the larger ducts, but remain separated from the epithelium by connective tissue.

In Figure 11 the cells are shown in close continuity with the duct epithelium, without intervening connective tissue. In these ducts, notably the intralobular and intercalary ones, the wall consists of two cell layers - an inner duct epithelium and an outer islet cell layer.

Figure 10.

Figure 10.

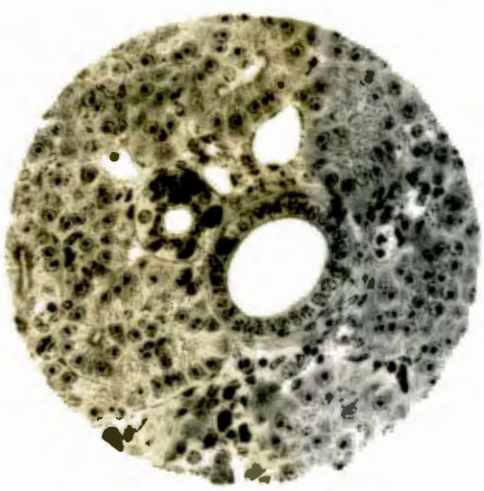


X 38.

Fixative: Zenker's Fluid,

Stain: Acid fuchsin methyl-green.

Figure 11.



X 38.

Fixative: Bayley's Fluid,

Stain: Azan (Haidenhain modification).

Sometimes the islet cells appear singly, or in varying numbers, interposed between the duct epithelium cells. It is interesting to note that these are mostly clear cells. They do not border the lumen but remain covered by a layer of cytoplasm from the neighbouring epithelial cells.

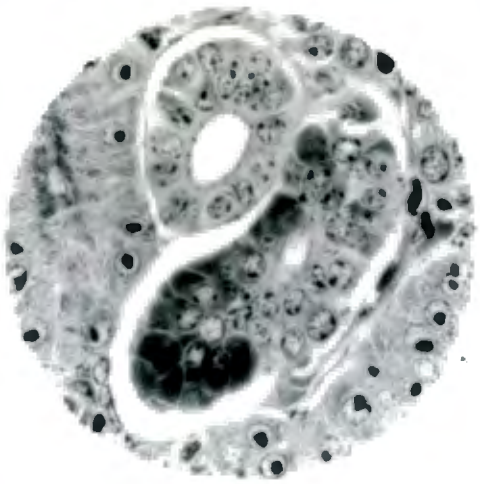
Figure 12.

Fixative: Orth's fluid,
Stain: Acid fuchsin methyl-green.

This observation seems to lend support to the suggestion that the clear cells are the most primitive type of islet cell, arising from the duct epithelium, and in its turn giving rise to A- or B-cells. Increasing numbers of islet cells in between the duct epithelium would then lead to their outward migration, thus forming a second layer surrounding the duct epithelium (Fig.11). Further migration away from the duct would lead to a solid cell cluster separated from the duct by connective tissue (Fig.10).

In other cases the islet cells surround what appears to be the remains of a duct without a trace of a duct epithelium. This is seen in Figure 13 and could represent a position where all duct cells had been transformed to islet cells with subsequent functional change as is evidenced by the decreased duct lumen.

Figure 13.

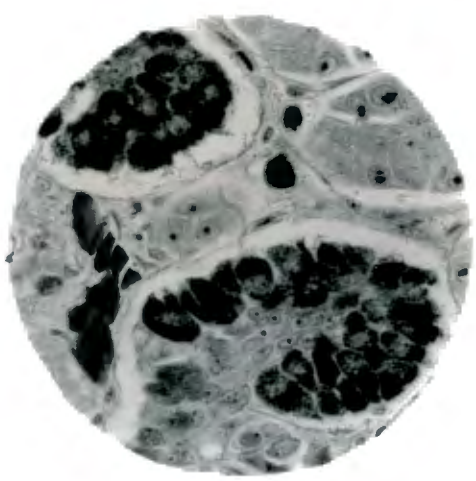


X 125.

Fixative: Susa.
Stain: Magenta blue.

By either of the above procedures solid clusters of islet cells, lying away from the ducts, but separated from the acinar tissue by connective tissue, originate. This condition is well illustrated in Fig.14:

Fig.14.

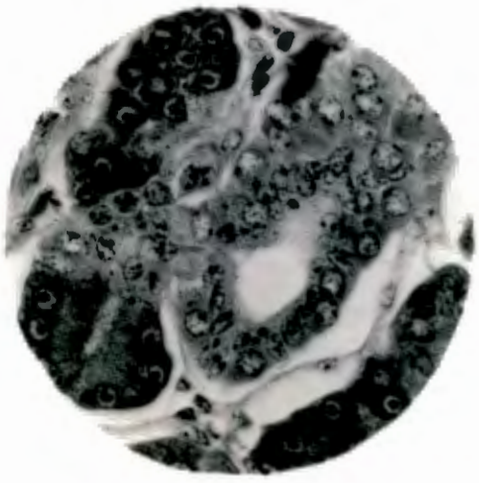


X 125.

Fixative: Lane's A.
Stain: Fuchsin blue.

However, this independence of islet tissue from acinar is not as complete as postulated by many investigators. In direct opposition to the theory of "independence" advanced by Bensley and others, islet cells are frequently found in close anatomical connection with acinar tissue. Figure 15 shows islet cells (A-type) apparently infiltrating an alveolus.

Figure 15.

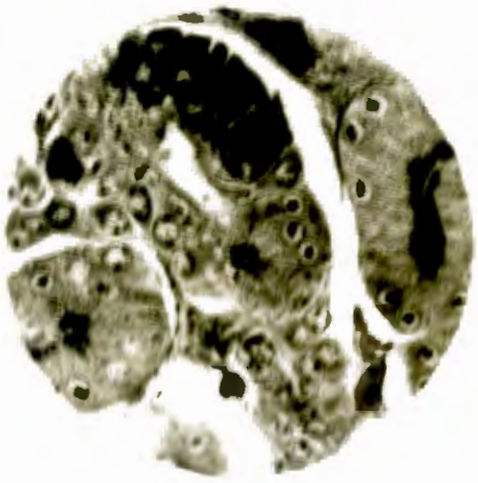


X 125.

Fixative: Zenker's fluid,
Stain: Eosin methylene blue.

At other times, as Fig. 16 shows, acinar cells appear incorporated in the island. The three acinar cells with their large, dark staining nucleoli can readily be distinguished.

Figure 16.

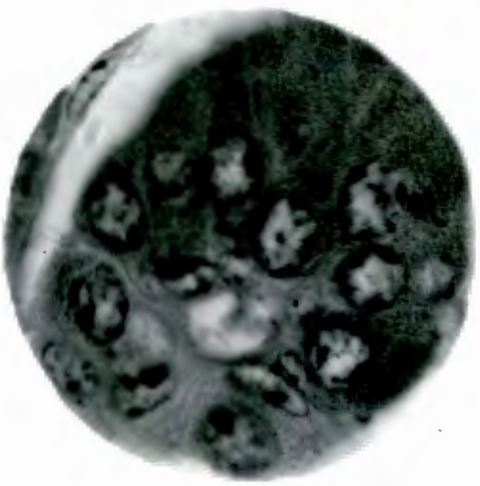


X 125.
Fixation: Zenker's fluid.
Stain: Acid fuchsin methyl-green.

With this observation is bound up the question of the acino-islet relationship. Regarding this, two schools have come to be established: one champions the idea of a fixed nature and non-interchangeability of the two tissues once they are fully differentiated, and the other favours a dynamic rather than static relationship, whereby transitions from acini to islets and vice versa can occur. Such a transition of endocrine into exocrine elements and vice versa in the mammalian pancreas has been described by Sergeyeva, 1940 (221). Lewaschew, 1886, in actuality, claimed the existence of "transitional cells", having described them as having both zymogenous and specific insular granules in the apical and basal portions of the cytoplasm respectively. No indication of such transition was found with this investigation . However, intimate connections between the two have been fully substantiated Due to the presence of single, scattered clear cells in the zymogenous tissue and of A-cells in continuity with acinar cells (Fig.15), it appears that, if this change does take place, it consists more of a transition from alveolus to acinus than of the reverse process.

As regards the granular cell types, the presence of only A- and B-cells is convincingly demonstrated. Figure 13 shows chiefly A-cells (light) surrounding a larger duct and B-cells (dark) surrounding the smaller lumen. The typical structure of the B-cells is shown in Figure 17.

Figure 17.

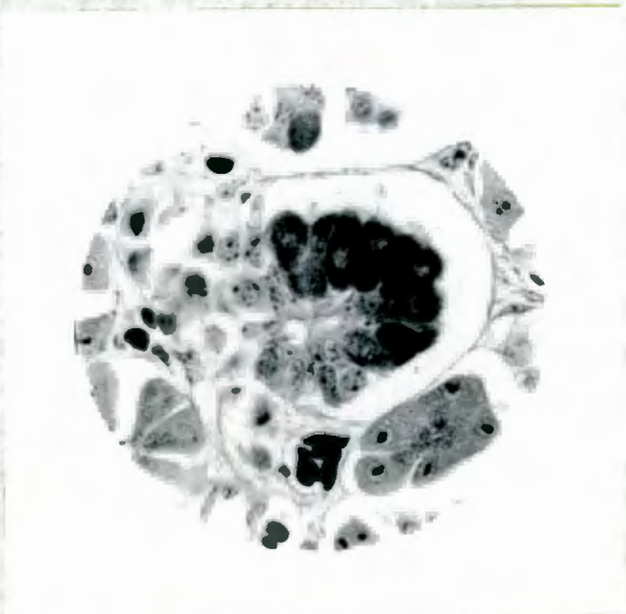


X 600.

Fixative: Zenker's fluid.
Stain: Haematoxylin and Eosin.

A-cells are well-illustrated in Figures 14 and 18.

Figure 18.



X 125. LANE'S A. ACID FUCHSIN METHYL-GREEN.

Of special interest are the large numbers of A-cells present in the shark pancreas. No explanation can be offered for the physiological significance of this.

Extraction of the pancreas yielded amounts of insulin as shown in Table 33:-

TABLE 33.

Yields of insulin from shark pancreas.

Serial No.	Insulin hydrochloride gm/200 gm. sample	Activity of hydrochloride I.U/mg.	Yield insulin hydrochloride per kilo material. I.U.
12	0.742	3.46	12,836
13	0.658	3.15	10, 364
14	0.672	3.54 3.13	11, 894 10, 516+
15	0.676	2.95	9,770
17	0.476	3.81	9, 068
20	0.608	3.64	11, 065.

+ As determined by full rabbit assay.

The results amply confirm the high insulin content of the pancreas in this elasmobranch species. Not only do the yields of crude hydrochloride equal or exceed those obtained from most other sources (0.440 gm./200 gm. material), but also the activity of the crude material is much higher than that found for any other source in this investigation. This again confirms what was stated previously, viz: that, using the picric acid-acetone method of extraction, the activity of the crude hydrochloride

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remains constant for a particular source (average 3.42 units/mg. for the shark), but it varies considerably from source to source (c.f. 0.40 unit/mg. for the Fin whale, 0.80 unit/mg. for the sheep, 1.20 unit/mg. for beef, 1.40 unit/mg. for pig, etc.). The potency of the hydrochloride obtained from a particular source, therefore, furnishes a fairly accurate indication of the insulin content of that source.

Calculated on the basis of 10,000 units per kilo of pancreas and a mean weight of 40 gm. per pancreas, the total amount of insulin to be derived from this source per month (i.e., 13,000) amounts to 5,200,000 units. This amount of insulin is obtained from approximately 8,000 head of cattle. Although the shark pancreas is not a particularly large organ, it is so firm and easily accessible, that it can be removed within a few seconds by even an unskilled worker. It is, therefore, clear that, wherever these fish are caught in large numbers (for other purposes such as oil, fertilizer, etc.), extraction of insulin from the pancreas could form a very profitable by-product.

The large amounts of islet tissue it contains can readily be seen from the sections on the preceding pages. The high proportion of A-cells has already been commented on. No attempt was made to determine the proportion of these to the B-cells, but it is evident that the percentage is much higher than that quoted for the dog (i.e., 25 per cent). The high insulin content of the shark pancreas is, therefore, difficult to reconcile with the elaboration of a blood sugar raising factor by a large part of its constituent cells.

(4) Conclusions.

The extremely primitive relationship of islet tissue to the duct system, is well illustrated in this elasmobranch species. Histological examination of this relationship furnishes evidence for the theory of islet tissue formation from the duct epithelium. In this process it appears that clear cells are first formed and these, in turn, give rise to the granular elements. No convincing evidence was obtained as regards the A-B cell relation, but it seems probable that the A-cells are the first to be formed from the clear cells and that these, in their turn, may give rise to B-cells. Sufficient evidence was obtained to constitute a definite acino-islet relationship, but whether this leads to a formation of islet tissue from acinar or vice versa is not clear. Available evidence favours the former process, in which case the A-cells were always observed to occupy a position in between acinar and B-cells.

On extraction, the pancreas yields from 10,000 to 12,000 units of insulin per kilo. This source is, therefore, worth exploitation wherever circumstances permit.

(3). INSULIN FROM SOUTH AFRICAN DOMESTIC ANIMALS.

(i) Introduction.

During 1949 the following numbers of animals were slaughtered in all Union abattoirs under the Union Meat Control Board: 1172,304 cattle, 2,822,580 sheep and 799,878 pigs (222). The Union of South Africa is one of the 34 countries which, as regards her insulin supply, is not self-sufficient but entirely dependent on import. From the larger centres as Cape Town, Johannesburg, Durban, etc. frozen pancreas is at present exported to Britain,

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but no material from the smaller centres is utilized for the purpose of insulin extraction.

This investigation was undertaken primarily with a view to obtaining a criterion (with the method of extraction used) by which the insulin yields from unknown sources (e.g., whale, shark) could be compared, and, secondarily, to establish the insulin content of the pancreas in South African domestic animals, thus obtaining an estimate of the annual insulin output to be expected if all material were to be utilized for extraction purposes.

All pancreases used in this investigation were obtained from the Capetown abattoir. They were removed as soon as the animals were killed and immediately rushed to the laboratory for extraction. Small samples of 200 gm. each were worked up according to the picric acid-acetone method, in addition to which one large sample (10-20 kg.) of each of beef, pig and sheep was extracted with acid alcohol and crystalline insulin prepared according to the method of Romano, Scott and Fisher (77). However, employing this method with fairly large samples in a laboratory which is not specially equipped for the purpose (i.e., with a sufficiently large basket centrifuge, pressure filters, vacuum still, etc.), is a difficult task. In order to effect distillation of the fairly large amounts of alcohol sufficiently quickly, distilling apparatus from commercial and other research laboratories was used. Apart from not being especially suitable for the purpose, much time was lost conveying materials from laboratory to laboratory, and the process further hampered by the fixed working hours of private institutions.
The overall effect of this was that, especially as far as the initial separation of the alcohol from the minced mass,

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the separation of the inactive precipitate at pH8 and the distillation of the alcohol were concerned, these processes could not be accomplished with the required effectiveness and speed. Nevertheless, crystalline insulin was successfully prepared in each instance.

(ii) Results and discussion.

The amounts of insulin extracted from beef, pig and sheep pancreas by means of the picric acid-acetone method are shown in Table 33:

Table 33.

Maximum Yields insulin hydrochloride obtained.

Source	Sample No.	Yield of insulin hydrochloride (crude) in gm./200 gm. sample	Potency of hydrochloride I.U./mg.	Insulin Yield (I.U.) per kilo of wet pancreas.
Beef	152	0.670	1.14	3819
	122	0.677	1.17	3960
			1.18	3062
	145	0.621	1.17	3632 †
	147	0.615	1.06	3260
Pig	82	0.694	1.32	4580
	83	0.740	1.28	4735
	84	0.654	1.41	4610
			1.22	4062
	85	0.666	1.30	4330 †
Sheep	71	0.646	0.62	2000
	72	0.584	0.83	2425
	73	0.642	0.58	1860
	74	0.612	0.66	2020

† as determined by mouse assay.

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The results of acid alcohol extraction and subsequent preparation of crystalline insulin are given in Table 34.

TABLE 34.

Source	Weight of material used (kg).	Weight of crystalline insulin (gm).	Activity of crystalline insulin I.U./mg.	Insulin Yield (I.U.) per kilo of wet pancreas.
Beef	10	0.928	20.4	1890
Pig	10	1.004	20.8	2088
Sheep	10	0.534	19.1	1020

Activity assayed by mouse method.

Photomicrographs of the crystalline material are shown in Figs.19, 20, 21.

Fig.19. Beef zinc insulin crystals. x600.

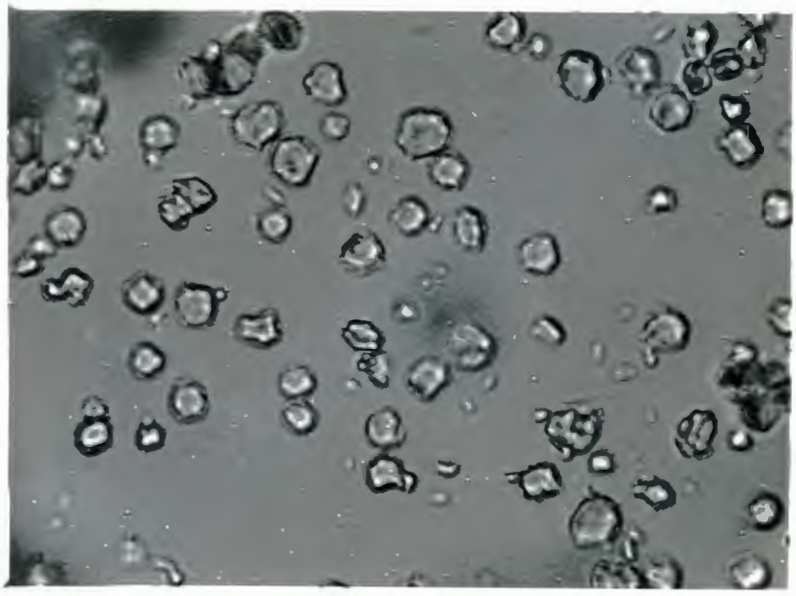


Fig.20. Pig zinc insulin crystals. x450.

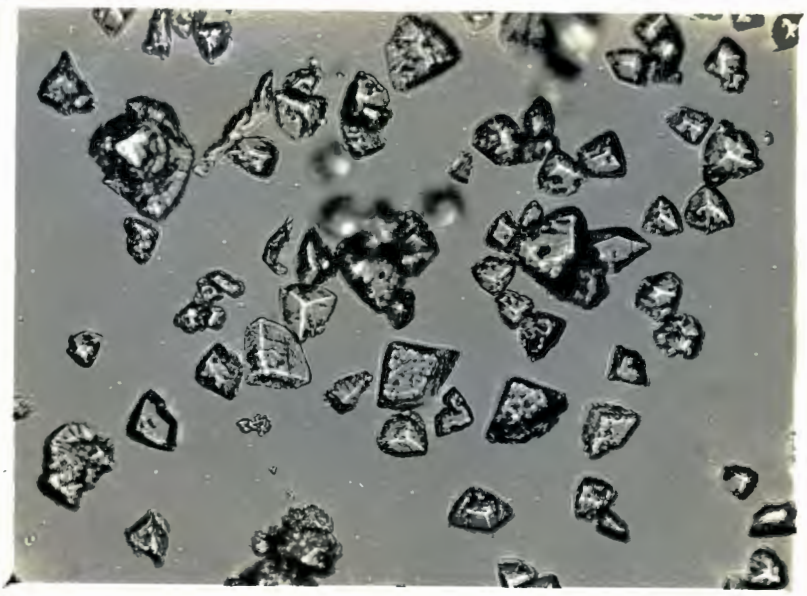
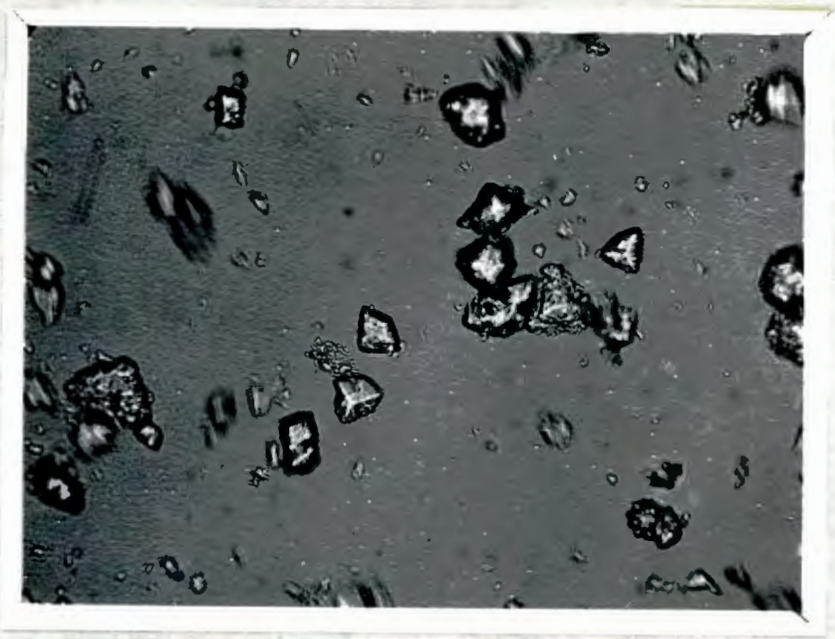


Fig. 21. Sheep zinc insulin crystals. X450.



The results of Table 33 indicate an insulin content of ± 3,500 units per kilo for beef pancreas, ± 4,500 for pig and ± 2,000 for sheep. These values are slightly in excess of those generally accepted for these sources, but in no way exceed claims that had been forthcoming for them in the past (c.f. 5,280 rabbit units per kilo. of pig's pancreas - Dodds and Dickens). The factors responsible for these high figures are twofold: Firstly, working with small samples and using the picric acid-acetone method, more insulin is extracted than with the acid alcohol method as it is generally described. This fact was convincingly shown by the comparatively higher yields obtained with this method by Dudley and Dickens and Dodds. Secondly, the material tested in each instance was the crude hydrochloride. It is generally agreed that purification is accompanied by loss of active material, so that the values given above would undoubtedly have been smaller had the hydrochloride undergone a process of purification.

This partly explains the discrepancy seen in the yields of hydrochloride (Table 33) and crystalline insulin (Table 34). Added to this is the fact already

/mentioned

mentioned that the acid alcohol extraction had not taken place under optimum conditions in a well-equipped (for the specific purpose) laboratory. Had this been the case, the values of Table 34 would probably have been markedly higher. On the whole, however, the claims of earlier investigators for the insulin content of the pancreas have been confirmed also for South African domestic animals. That the sheep pancreas is not as profitable a source for commercial extraction purposes is fully borne out by the results.

If the average weight of the ox pancreas is considered as 300 gm. and that of the pig as 120 gm., then the total amount of insulin to be derived from these two sources, if all material were utilized, would be 900,000,000 and 288,000,000 units respectively, totalling 1,188,000,000 units annually (calculated at 2,500 units per kilo of beef pancreas and 3,000 per kilo for the pig). No exact information was available on the Union's import demands for insulin, but it is clear that the above figure exceeds by far the country's demand. From these sources, together with that obtainable from the fishing industry and the state-controlled seal industry, amounts of insulin sufficient for the country's own use plus an exportable surplus could be produced (no information is at present available on the insulin content of the seal pancreas, investigation of this source being held in prospect).

From Figures 19, 20 and 21, it is evident that the zinc insulin crystals prepared from pig and sheep are similar in appearance (i.e, wedge-shaped) and of the same size. Those from beef, on the other hand, are micro-crystals and rhombohedral. On two successive occasions this same type of crystals was obtained from this source.

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Yet, the procedure followed was exactly the same in each instance. It seems, therefore, that other factors, beside the pH at which crystallisation is effected, are concerned with the ultimate shape and size of the crystals.

SUMMARY.

1. The effect of graded doses of insulin (0.05-0.85 unit/2 kg., varying with 0.05 unit) on the blood sugar of fasting rabbits has been studied.

2. Evidence was obtained that small doses of insulin show a well-graded blood sugar response.

3. The dosage-response curves and the dosage-response lines were drawn by plotting response against dosage and against the logarithm of the dose respectively.

4. The dosage-response curves show characteristic flattening at the higher and lower dosage levels; these have been argued to be due to too long a period over which effect is measured and too short a period respectively. Adjustment of the period of testing and the bleeding schedule followed to the dose would result in proportionality of dose-response over a much wider range.

5. The effect of these doses can be determined by measuring (i) the average absolute fall over a period of three hours, or (ii) the average absolute fall at the end of the second hour, or (iii) the average absolute blood sugar level at the end of the second hour.

6. Calculation of the standard deviation and the coefficient of variation in each instance indicates that, of the three procedures, the last gives the most reliable results. In a similarly treated group of rabbits, the initial sugar value can be discarded and calculations based on the absolute blood sugar level, two hours after injection, only.

7. It is suggested that, in the assay of insulin, the blood sugar level is a more accurate measurement of effect than the fall of the blood sugar (expressed either as

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absolute or percentage).

8. A coarse method of assay to be used where large numbers of unknown samples are concerned, is proposed and discussed. The basis for this method is a predetermined log.dose - response curve, plotting the absolute blood sugar level two hours after injection against the logarithm of the dose.

9. The picric acid-acetone method of insulin extraction has been fully investigated.

10. Acetone has no specific solubility for insulin picrate as such, but rather for the entire complex of accompanying protein picrates. The optimum concentration of acetone for this purpose was found to be about 80 per cent.

11. The insulin hydrochloride so obtained is a crude product, the activity of which varies with the source from which it is derived. The activity of the hydrochloride furnishes a fairly accurate estimate of the insulin content of the source.

12. The effect of different factors such as time of fixation, acetone, conversion of the picrate to the hydrochloride, stability of the hydrochloride, etc. was investigated. The most outstanding feature of the method is the stability of the picrate.

13. The yield of insulin by this method exceeds that of the acid alcohol method. For experimental work and extraction on small scale it is to be recommended.

14. The use of picric acid as such, with its staining and other specific qualities, is the chief disadvantage of the method.

15. The insulin content of the pancreas in the whale, "vaalhaai" and South African cattle, pigs and sheep has been investigated.

/16.

16. The pancreas in the Blue whale, Fin and Humpback is naturally poor in insulin, yielding from 1,000-1,500 units per kilo of material. That of the Sperm, on the other hand, is much higher, approximating beef pancreas. On the whole, commercial extraction of insulin from this source cannot be profitably undertaken. Added to this is the rapid loss of insulin which occurs chiefly as a result of the method of killing the animals.

17. A study of the histological structure of the pancreas in the "vaalhaai" was undertaken. This elasmobranch species furnishes supporting evidence for the formation of acinar and islet tissue from the tubular epithelium.

18. The shark pancreas has a high insulin content - 10,000-12,000 units per kilo. Where these fish are caught in large numbers, insulin could constitute a valuable by-product.

19. The claims of earlier investigators regarding the insulin content of the pancreas in cattle, pigs and sheep have been confirmed.

20. Pooling of all raw material in South Africa could result in the production of sufficient amounts of insulin for the country's consumption plus an exportable surplus.

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J.

K.

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O.

P.

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S.

T.

U.

V.

W.

Y.

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