EFFECTS OF INSULIN ON BLOOD GLUCOSE ENTRY AND REMOVAL RATES IN NORMAL DOGS*

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Despite considerable efforts over many years, the sites and mechanisms of insulin action remain uncertain. Although almost every conceivable step in glucose catabolism has been implicated, from its initial entry into the cell to its terminal stages of oxidation, it has not yet been established which of these so called insulin effects are primary or secondary, or how closely they are related to the physiological functions of the hormone (1–4).

The most immediate and consistent known effect of insulin is the lowering of the blood sugar of the intact animal. The blood sugar of the post-absorptive animal is maintained constant by a balance between entry¹ of new glucose molecules, presumably from the liver, and removal¹ by peripheral tissues. When insulin lowers the blood sugar, it must do so either by stimulating removal of glucose or inhibiting its entry, or by doing both. At the time the present study was undertaken, no decisive information was available to indicate how much of the hypoglycemic action of insulin was due to either of these possible actions. In the present report we wish to describe experiments which clearly show that insulin exerts its hypoglycemic action both by inhibiting the entry of new glucose molecules into the blood and by accelerating their removal.

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¹ The term “entry,” as used in this paper, refers to the introduction of newly synthesized glucose molecules into the blood stream or such extracellular fluids as are in rapid equilibrium therewith. No commitment is made regarding the source, but the assumption is made that the liver is essentially the only organ which contributes glucose to the blood. The term “removal” refers to the glucose disappearing from the blood, again without commitment concerning the organs involved.
EFFECTS OF INSULIN ON BLOOD GLUCOSE

The procedure used represents an extension of a familiar isotope tracer technique employed previously for studies of glucose "turnover" in normal, diabetic, and adrenalectomized rats (5-7), in normal and diabetic dogs (8, 9), and in normal and diabetic humans (10, 11). A "trace" dose of uniformly C\(^{14}\)-labeled glucose is injected and blood samples are removed at intervals for determination of blood sugar content and specific activity. The initial dilution of specific activity allows the calculation of the glucose pool size, and the logarithmic drop in specific activity, with time, allows the calculation of turnover rate from the first order reaction rate

\[ R = 2.3(b/t) \log \frac{i_0}{i_t} \]

where \( R \) = the turnover rate; \( b \) = the glucose level; and \( i_0 \) and \( i_t \) = the initial and final specific activities of glucose.

The departure from this conventional procedure in the present study consists in administering insulin after sufficient samples have been collected to calculate the turnover rate. It is well established that insulin injection will result in a rapid fall in the blood sugar level, followed by a variable hypoglycemic period and a slow rise to normal. If the initial fall in blood sugar arises primarily from an acceleration of removal, the specific activity will continue to fall as rapidly as before; however, if the drop is due in some part to an inhibition of entry, the specific activity will fall less rapidly, since the blood glucose will be more slowly diluted by new, unlabeled molecules. It was felt that this simple procedure could give a clear cut indication of the immediate action of insulin before it would be obscured by countereffects of other hormones. One requires only the reasonable assumption that any new glucose molecules entering the blood will be predominantly unlabeled.

The principle of this procedure has already been applied by Searle and Chaikoff (12) in establishing that entry of glucose into the blood of normal dogs is completely inhibited during hyperglycemia.

The data obtained in such experiments are also amenable to quantitative evaluation. When the blood sugar is constant, rates of entry and removal are equal, and can be calculated from the fall in specific activity by using Equation 1. When the blood sugar level is changing, as during hypoglycemia, the rates of entry and removal will be different; under these circumstances it is felt that this assumption is a reasonable one. We have consistently observed a strictly linear drop in the logarithm of the specific activity of the blood glucose in the fasting dog, indicating a constant entry of unlabeled glucose. It is conceivable that some of the labeled glucose which disappears may be converted to glycogen and may subsequently reenter the blood. However, glycogen storage in the fasting animal would not be expected, and, even if this did occur, it seems likely that insulin would enhance rather than reverse this process.
cumstances another set of equations, Equations 2 and 3, can be used,

\[ R_1 = \frac{(B_0 - B_t) \log i_0/i_t}{t \log B_0/B_t} \]  
(2)

\[ R_2 = (B_0 - B_t) + R_1 \]  
(3)

where \( R_1 \) is the rate of entry, \( R_2 \) is the rate of removal, \( B_0 \) is the initial glucose level, \( B_t \) is the level at time \( t \), and \( i_0 \) and \( i_t \) are the initial and final specific activities. These equations were originally applied to the calculation of ketone body "turnover" rates in tissue slices (13, 14). It was anticipated that these equations would be used for calculation of blood glucose entry and removal rates during the initial stages of insulin hypoglycemia when the level is changing rapidly. As it happened, during this initial hypoglycemia the entry rate, \( R_1 \), was zero, thus obviating the necessity for using these equations. However, they were applied in calculating turnover during the later stages of hypoglycemia and during recovery, so as to obtain a complete picture of entry and removal throughout the duration of hypoglycemia.

While this work was in progress, two somewhat similar investigations were published. Wrenshall (15) developed a method of computing rates of transfer of components in and out of a central body compartment such as the blood, and has applied this to a study of the action of insulin on blood glucose transfer in the depancreatized dog (16). With use of a method involving continuous intravenous infusion of glucose-C\(^{14}\), Wall et al. (17) reported on the effects of insulin and other hormones on turnover rates of blood glucose in dogs.

**Methods**

An initial 2 ml. blood sample was obtained from one of the external jugular veins, and a trace dose of uniformly labeled C\(^{14}\)-glucose, representing approximately 1.5 to 2.0 per cent of the total body pool, was injected. Blood samples of 2 ml. each were collected at approximately 15 minute intervals for 45 to 60 minutes from the time of labeled glucose administration. This represented the control period for each dog. At this time, 3 to 10 units of insulin were injected and blood samples were obtained at 5 to 10 minute intervals for the 1st hour and at 20 to 30 minute intervals thereafter for a total of approximately 4 hours. Usually sixteen to twenty

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3 This procedure was originally applied to rabbits (18). To obtain sufficient blood at frequent intervals, it was necessary to anesthetize the animals and cannulate a femoral vein. Because of the hyperglycemic effects of the Nembutal used for anesthesia, the results were often obscured by pronounced rises in the blood sugar prior to insulin injection. In the successful experiments, however, exactly the same pattern of response was obtained as is reported in the present experiments with unanesthetized dogs.
samples were collected. The animals were accustomed to experiments of this type and at no time offered any resistance to venipuncture. During the length of the experiments the dogs were not restrained but were allowed to move about freely within the confines of a large hood, with access to water.

The blood samples were deproteinized by the method of Somogyi (19), and 1 to 2 ml. aliquots of the resulting protein-free filtrates were analyzed in duplicate for glucose by the anthrone method (20). The remainder of the filtrate was used for radioactivity assay.

**Radioactivity Assay**—The isolation of glucose carbon is based on the action of periodic acid on glucose to yield 5 molecules of formic acid and 1 of formaldehyde (21). The formic acid is oxidized to carbon dioxide by the specific action of mercuric ions in acid solution, and is recovered and counted as BaCO₃. The formaldehyde may be precipitated with dimedon (22) and counted as formaldemethone. The stoichiometric nature of the reaction was established by quantitative recoveries of formic acid and formaldehyde from standard glucose solutions, and the absence of appreciable quantities of interfering substances in the blood filtrates was established.

In the earlier experiments with rabbits (18), carbon 6 was obtained and assayed separately as formaldemethone. Since it invariably had the same activity as the formic acid derived from carbons 1 to 5, this step was omitted in subsequent experiments. However, both procedures are described.

**Reagents**—
1. Periodic acid. 7 gm. of H₃IO₆ (obtained from the G. Frederick Smith Chemical Company, Columbus, Ohio) are dissolved in water and made up to 100 ml.
2. Standard glucose solution. Exactly 1.0 gm. of anhydrous glucose is dissolved in water and made up to 100 ml.
3. Sodium bicarbonate, 1 M.
4. Hydrochloric acid, 1 M.
5. Sodium arsenite, 1.2 M.
6. Sodium acetate, 1 M.
7. Dimedon, 80 mg. per ml. of 95 per cent ethanol.
8. Mercuric sulfate solution, 10 per cent. 73 gm. of red mercuric oxide are dissolved in 1 liter of 4 N sulfuric acid.
9. Sulfuric acid, 50 per cent by volume.
10. Sodium hydroxide, 0.5 M CO₂-free.

**Oxidation of Glucose**—5 ml. of the protein-free blood filtrate, representing 0.5 ml. of blood containing up to 100 μmoles of glucose, in a 125 ml. Erlenmeyer flask are mixed with 1 ml. of the standard glucose solution
containing 10 mg. per ml. 2 ml. of 7 per cent periodic acid solution and 2 ml. of 1 M NaHCO₃ solution are added, and the contents of the flask are mixed thoroughly and allowed to stand at room temperature for at least 1 hour. Excess periodate is then destroyed by addition of 3 ml. of 1 M HCl, followed by 2 ml. of 1.2 M sodium arsenite solution. The reaction mixture is then allowed to stand at room temperature until the precipitate and yellow color disappear.

Precipitation of Formaldehyde with Dimedon (Optional)—To the contents of the reaction flask are added 2 ml. of 1 M Na acetate solution and 1 ml. of dimedon solution. The flask is heated in a boiling water bath for 10 minutes, then allowed to stand at room temperature for 1 hour. The dimedon precipitate is then filtered, weighed, and counted as such. The product may be recrystallized by dissolving in a small amount (approximately 5 ml.) of acetone and reprecipitated by addition of water (m.p., 189–190°).

Oxidation of Formate (If carbon 6 is not to be obtained as formaldehyde, this part is conducted directly on the periodate reaction mixture.) The apparatus used is similar to that described for oxidation of labeled compounds by persulfate (23). The contents of the reaction flask are washed into the persulfate flask, the volume is made up to 30 ml., and 2 ml. of 50 per cent H₂SO₄ are added. The apparatus is assembled as shown by Calvin et al. (23), and aeration with CO₂-free air is carried out for 10 minutes to insure removal of CO₂. This is done by application of suction at the top of the bead tower. 10 ml. of 0.5 m CO₂-free NaOH are then added to the Erlenmeyer flask attached as shown, below the bead tower, and 15 ml. of 10 per cent HgSO₄ are added to the formate solution. Air is then drawn through the flask and bead tower for 30 minutes while the solution is heated to boiling and allowed to reflux. The Erlenmeyer flask is disengaged, and the trapped CO₂ in the bead tower is washed down into the flask with three 10 ml. portions of hot CO₂-free water. The carbonate is precipitated therefrom by addition of 3 ml. of 20 per cent BaCl₂ solution, and counted by conventional procedures with a thin window Geiger tube.

In the experiments reported here, all radioactivity measurements have been corrected for self-absorption to a layer of infinite thickness, and for the added carrier glucose, and are expressed as counts per minute per standard dish of 7.5 sq. cm. area. The values reported are averages of duplicate determinations which agree within 10 per cent.

The uniformly C¹⁴-labeled glucose was obtained from the Nuclear Instrument and Chemical Corporation upon allocation by the United States Atomic Energy Commission. The insulin, free from hyperglycemic factor, was obtained through the kindness of Eli Lilly and Company.
Results

Fig. 1 shows the results of a typical experiment. 55 mg. (101 μc.) of glucose, with a specific activity of 1.1 × 10⁶ c.p.m., were injected into a male dog weighing 11.7 kilos. During the 30 minutes between injections of glucose and insulin, the blood sugar remained relatively constant between 118 and 127 mg. per 100 ml., while the specific activity fell from an extrapolated initial value of 12,300 to 8600 c.p.m. From the dilution of glucose specific activity, the glucose pool size is estimated to be 1,100,000 × 55/12,300 = 4940 mg. From the average glucose concentration of 122 mg. per 100 ml., we can calculate a glucose space of 4940/1.22 = 4030 ml., which is 4030/11,700 = 34 per cent of the body weight. From the drop in specific activity, the glucose turnover rate was calculated, from Equation 1, to be 1.4 mg. per 100 ml. per minute, or 4.8 mg. per kilo per minute. These values are in the range reported previously by other investigators (8, 9, 16).

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Upon administration of 10 units of insulin, the blood sugar fell promptly. In 9 minutes it had dropped to 92 mg. per 100 ml., and in 20 minutes it reached the minimum of 64 mg. per 100 ml. During this period of maximal blood sugar removal there was essentially no change in the specific activity of the blood glucose; this indicates that glucose was not entering the blood.
At the time coinciding with the "leveling off" of the blood sugar content, the specific activity resumed its downward trend, and while the blood sugar gradually rose to the normal level, its specific activity continued to drop, but at a gradually reduced rate.

Fig. 2. Time-course of blood glucose entry and removal rates as affected by insulin administration. Entry rate, \( R_e \), solid line; removal rate, \( R_a \), broken line. The values are given in mg. per 100 ml. per minute. A, Experiment 1, shown in Fig. 1; B, Experiment 3, shown in Fig. 4; and C, Experiment 2, shown in Fig. 3.

Since glucose was not entering the blood during the initial hypoglycemia, we can calculate very simply the effect of insulin on glucose removal, from the rate of drop in blood sugar level. During the first 15 minutes of hypoglycemia, the blood sugar dropped from 118 to 73 mg. per cent, representing an average rate of removal of 3 mg. per 100 ml. per minute or 11.1 mg. per kilo per minute. This is almost 3 times the original turnover rate before injection of insulin. The resumption of a downward trend in the specific activity of the blood glucose indicates that, after the initial phase of hypoglycemia, glucose again enters the blood, and this is reflected in a decrease in the rate at which the blood sugar is falling. During the next
5 minutes, in which the blood sugar fell from 73 to 55 mg. per 100 ml.,
average rates of entry and removal were calculated from Equations 2 and
3. During the period of relative constancy in level, between 50 and 105
minutes, the blood sugar was assumed to be constant at the average value
of 60 mg. per 100 ml., and "turnover" was calculated from Equation 1.
During the recovery phase, from 105 to 180 minutes, Equations 2 and 3
were again used. The rates thus calculated are presented graphically in
Fig. 2, A. Though they are regarded as only rough approximations, they
provide a clear insight into the immediate effects of insulin. The most
pronounced effect is the rise in removal to twice the original rate. After
10 minutes it reached 3 times the original rate, but in 15 minutes it was
back to the initial value and remained essentially constant throughout the
hypoglycemia and recovery periods. The other immediate response was
the complete inhibition of glucose output. This was reversed after 15
minutes and reached essentially the initial value after 20 minutes, at which
it remained throughout the hypoglycemic interval. During the recovery
period the entry rate was slightly higher than the removal rate; this small
difference accounts for the gradual rise to normal glucose level between
105 and 180 minutes.

The data on a similar experiment with a female dog of similar weight are
given in Fig. 3 and Fig. 2, C. Essentially the same picture appeared. In this experiment, the postabsorptive blood sugar dropped somewhat during the preinsulin period, resulting in slight separation of the entry and removal rates, as shown in Fig. 2, C. Again, when insulin was given, the two rates separated widely; as the entry dropped to zero, the removal more than doubled, then quadrupled its original rate. In 20 minutes, both rates had converged to their original values, at which they remained during the rest of the hypoglycemic and recovery periods.

Fig. 4. Same experiment as in Fig. 3, with the same dog, weighing 11.8 kilos, except that 3 units of insulin were administered 60 minutes after glucose administration.

In the same dog, an experiment was performed with a much smaller dose of insulin; i.e., 3 instead of 10 units. Here, the drop in blood sugar was not so great and the recovery was much more rapid (see Fig. 4 and Fig. 2, B); however, the specific activity behavior was quite similar. Again, a plateau appeared, following which the specific activity dropped rapidly during the hypoglycemia and recovery, then continued to drop at a lower rate during the period of blood sugar constancy after recovery. As shown in Fig. 2, B, the rates of entry and removal were at 1.1 mg. per 100 ml. before insulin; upon insulin injection, the removal rate approximately doubled, remained high for 25 minutes, then fell to about the initial value. Meanwhile, the entry rate fell to zero for 10 minutes, then gradually rose to more than twice the normal rate during the recovery phase, and finally leveled off at near the initial rate.
DISCUSSION

Using the technique of hepatic venous catheterization in normal and diabetic humans, Bearn, Billing, and Sherlock (24) found that insulin injection caused an immediate drop in the hepatic glucose output. The present results are in complete accord with these findings. While the work reported here was in progress, two other studies on glucose turnover in dogs appeared, which are essentially in agreement with our findings, and which also confirm and amplify the conclusions of Bearn et al. (24).

Henderson et al. (16), in experiments similar to those reported here, found that the depancreatized dog responded to insulin injection by markedly increasing its disappearance rate of plasma glucose, from about 5 mg. per kilo per minute to about 12 mg. per kilo per minute. At the same time there was a diminution of glucose output which, however, in contrast with our results on normal dogs and with those of Bearn et al. in diabetic humans (24), was of minor magnitude and developed more slowly. In a report available in abstract form, Wall et al. (17) calculated plasma glucose inflow and outflow rates in dogs, using continuous intravenous infusion of glucose-C\(^{14}\). They found, as we did, that insulin administration resulted in an initial decrease of inflow, but that the hypoglycemic response was primarily owing to increased outflow.

These isotope tracer studies, which clearly indicate an action of insulin in inhibiting hepatic glucose output, may be added to a growing body of information which is making it increasingly obvious that a major action of insulin is exerted on the liver. It is already well recognized, as a result of hepatic venous catheterization, that net hepatic glucose output is diminished during hyperglycemia in dogs (25, 26) and in man (27, 28). In subsequent studies on glucose turnover in normal dogs with C\(^{14}\) labeling, Searle and Chaikoff (12) strikingly demonstrated that entry of glucose into the blood is completely inhibited during hyperglycemia. This observation has been confirmed in numerous as yet unpublished experiments from our laboratory.

On the other hand, the diabetic animal secretes glucose into the bloodstream during hyperglycemia. Despite the difference in blood glucose levels, the net hepatic glucose output in diabetic humans (28–30) and in depancreatized dogs (26) is at least as high as in the normal counterparts. In glucose turnover studies, using C\(^{14}\) labeling, Searle et al. (8) and Feller et al. (9) found glucose turnover in depancreatized dogs to be somewhat higher than in normal ones, indicating a higher hepatic glucose output; similar observations were made in rats by Feller et al. (5) and Welt et al. (6). Henderson et al. (16) also reported values for glucose turnover in depancreatized dogs similar in magnitude to those reported in these previous studies and in the same range observed by us in the present study.
The fact that the diabetic animal adds glucose to the blood at hyperglycemic levels has long been recognized by Soskin and Levine (1) p. 277 and is the keystone of their "overproduction theory" of diabetes. It appears to us that our present observations, taken in conjunction with those of Bearn et al. (24), Wall et al. (17), and Henderson et al. (16), confirm and extend these earlier findings by demonstrating that insulin does in fact act immediately to inhibit glucose production by liver. Since insulin exerts this action in both hyperglycemic and hypoglycemic states, it seems reasonable to assume that the diabetic hyperglycemia is, in part at least, directly due to inability of the animal lacking insulin to control hepatic glucose output.

It is interesting that the present study, as well as those of Henderson et al. (16) and Wall et al. (17), also confirms what has hitherto been regarded as an opposing theory of insulin action; namely, that it increases glucose utilization. The obvious answer seems to be that insulin does both. At present it is not entirely certain what are the relative roles of liver and peripheral tissues in the insulin-stimulated glucose utilization. Though classically the primary action has been assumed to be on the periphery, the recent reports by de Duve and his associates (31, 32) leave no doubt that the liver plays a major role in glucose utilization by the hyperinsulinized dog. Bearn et al. (24) also observed, under certain circumstances, removal of glucose by liver under insulin action in humans. Thus, in at least one tissue, liver, insulin appears to cause an actual reversal in glucose flow.

An action of insulin in increasing the utilization of glucose by liver is becoming increasingly evident also from studies in vitro. The marked impairment of lipogenesis from glucose or its intermediary metabolites, such as lactate or acetate, in liver slices of the alloxan-diabetic rabbit, and its restoration by pretreatment of the animals with insulin (33-35), pointed to a role of glucose catabolism in fatty acid synthesis. Although a primary role of insulin was discounted, because of the necessity of pretreating for 24 hours or longer in order to observe this effect, Haft and Miller (36) recently reported an immediate effect of insulin on lipogenesis in the perfused rat liver. More recently, Berthet et al. (37) clearly established an enhancement of glycogen formation from glucose-C^{14} in rabbit liver slices by direct addition of insulin in vitro.

At present no information is available to indicate precisely how insulin regulates the flow of glucose in and out of cells. It is becoming increasingly evident, from recent studies of Levine and Goldstein (3) and Park (38), that insulin accelerates the entry of sugars into cells; the studies of Stadie (4) suggest that this occurs by way of a fixation of insulin to the cell membrane. The present study, in the light of the foregoing discussion,
suggests that such a combination of insulin with the cell membrane might also prevent the flow of glucose out of cells. Such a dual action of insulin at its site of binding to the cell membrane does not appear unreasonable, and would go far toward reconciling experimental data and theories hitherto regarded as controversial.

SUMMARY

An isotope tracer procedure was applied to determine how much of the hypoglycemic action of insulin is due to inhibition of entry and how much to stimulation of removal of blood glucose. Unanesthetized, fasting dogs were given a trace dose of glucose-$^{14}C$. Blood samples were then removed at frequent intervals before and after intravenous administration of insulin, and were assayed for glucose content and specific activity. Before insulin injection, the glucose level remained constant and its specific activity fell logarithmically, indicating a constant "turnover" of from 2 to 5 mg. per kilo per minute. Immediately after insulin, the specific activity reached a plateau and remained constant throughout the initial hypoglycemic phase lasting 10 to 20 minutes. As the blood sugar leveled and slowly rose, the specific activity resumed the downward trend. From one-fourth to one-half of the total drop in blood sugar was estimated to be due to inhibition of entry and the remainder to increased removal. It is suggested that the immediate inhibition of blood glucose entry and acceleration of removal may arise from a dual action of insulin, bound to the cell surface, in favoring the inward and preventing the outward flow of the sugar.

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