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ABSORPTION OF ORALLY ADMINISTERED INSULIN BY THE NEWLY BORN CALF

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Previous studies (Pierce, 1959, 1961; Pierce & Johnson, 1960) have shown that the proteinuria of the new-born calf arises mainly from the excretion, via the kidney, of the low-molecular-weight proteins of colostrum which are absorbed from the gut together with immune lactoglobulin. These low-molecular-weight proteins are rapidly removed from the circulation, probably by glomerular filtration. Their concentration in the blood is consequently never sufficiently high for them to be readily detected, either electrophoretically or by use of the analytical ultracentrifuge, although they can be detected by immunological techniques. The immune lactoglobulin, on the other hand, has a molecular weight of 180,000, is not cleared by the kidney, and therefore accumulates in the circulation, reaching amounts which can be readily detected.

It is in relation to the absorption of colostral proteins that further evidence of the fate of orally administered low-molecular-weight proteins has been sought by studying the absorption of bovine insulin in the newborn calf. This homologous protein is comparable in size with the low-molecular-weight β-lactoglobulins in colostrum and is readily detected by its hormonal activity or by immunological techniques. Preservation of biological activity would also indicate that the protein had not been altered during absorption.

There is some evidence that bovine insulin can pass through the intestinal wall of the infant rat (Mosinger, Placer & Koldovsky, 1959), the infant mouse (Kelly, 1960) and the newly born pig (Asplund, Grummer & Phillips, 1962), when given by stomach tube in doses which are far in excess of the parenteral dose required to produce equivalent hypoglycaemic responses in these animals. It is also of interest to know the route by which absorbed proteins enter the general circulation. Earlier studies (Pierce & Johnson, 1960; Pierce, 1962) have confirmed those of Comline, Roberts & Titchen (1951), showing that immune lactoglobulin is absorbed via the lymphatic system. On the other hand, Balfour &

Comline (1959) have presented evidence which suggested that low-molecular-weight proteins might pass directly into the blood rather than into lymphatic vessels. With a molecular weight of ~ 36,000 assuming the molecule to be a hexamer, insulin is smaller than serum albumin (~ 69,000) (Scatchard, Batchelder & Brown, 1946; Rowe & Abrams, 1957) and therefore, if absorbed at all, it might also pass directly into the blood.

METHODS

Preparation of insulin and management of calves. Commercial crystalline bovine insulin (anhydrous potency 25 u./mg) was used. Stock solutions of the insulin were prepared by adding N-HCl to a suspension of insulin in water until solution was obtained. After adjusting to an appropriate concentration the solution was filtered and dispensed into small vials; the pH of stock solution thus prepared was 3·4. These vials were stored at 4° C until required. For intravenous injection a suitable dilution of the stock solution was prepared. For oral administration the contents of several vials were added to the feed to give the required dose. In the experiments first carried out (calves T 14, T 18, U 3, U 4) feeds were sucked by the calf through an artificial teat; thereafter all feeds were given through a stomach tube. The volume of all feeds, with or without insulin, was 20 ml./kg body weight. Nineteen calves were used in the present work.

The management of the calves, the collection and storage of serum and urine samples have all been described previously (Pierce, 1959). All absorption experiments were carried out within the first 24 hr after birth. Blood, for glucose determination, was collected directly from the jugular vein into oxalated vessels which were immediately sealed and kept at −10° C until the assays were carried out, usually within 4 days.

The collection of lymph. The calf was anaesthetized with intravenous pentobarbitone. Anaesthesia was maintained throughout the experiment by using cyclopropane in a closed circuit system. Lymph was collected through a cannula placed in the thoracic duct, by the operative technique of Lascelles & Morris (1961). Lymph samples were taken before and after the administration of insulin (500 u./kg body wt.) in sodium bicarbonate solution 5 g/100 ml., 20 ml./kg body wt., through a cannula placed in the duodenum.

Glucose determination. Frozen blood samples, allowed to thaw at room temperature, were deproteinized by the addition of barium hydroxide and zinc sulphate solutions (Hawk, Oser & Summerson, 1954). After filtration, glucose was determined by the glucose oxidase method of Huggett & Nixon (1957). No difference was observed between control blood samples assayed fresh and following thawing after storage at −10° C.

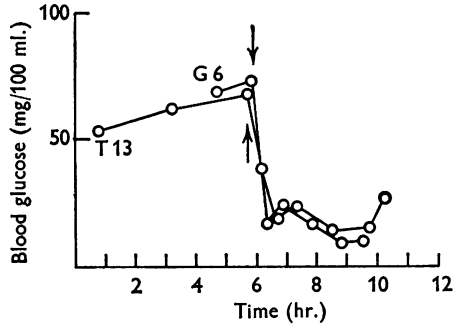
Immunological tests for insulin in urine. Urine samples were concentrated either by dialysis against high-molecular-weight polyethylene glycol (Kohn, 1959) or by dialysis against tap water followed by freeze-drying; they were then examined by the immunological technique of Ouchterlony (1953), precipitating sera produced in guinea-pigs against bovine insulin being used (Birkinshaw, Randall & Risdall, 1962). These tests were carried out in plates of agar poured in Petri dishes or in thinner films of agar poured on 3 × 1 in. (76 × 25 mm) microscope slides. The lines of precipitate were recorded photographically either by means of dark-ground illumination or by enlarging directly from washed and dried agar films stained with naphthalene black (Hutchinson, 1962).

Tests for insulin-like activity. The insulin-like activity in blood was assayed by the method described by Stewart & Young (1963) with rat adipose tissue, or by the radioimmune technique of Hales & Randle (1962). The lymph samples were examined by the latter technique only. Insulin, obtained from some samples of urine, was assayed by a mouse convulsion method based on that described in the British Pharmacopaeia (1958).

RESULTS

The effect of insulin on blood glucose

Insulin was given intravenously to two calves. One (T 13) received a dose of 2 u./kg and the other (G 6) 4 u./kg. The results are shown in Text-fig. 1. After the injection the blood glucose level fell rapidly, reaching a value of less than 20 mg glucose/100 ml. blood. Both calves showed signs of increasing distress during the experiment. Glucose was administered intravenously immediately after the last blood sample had been taken; the calves recovered rapidly.



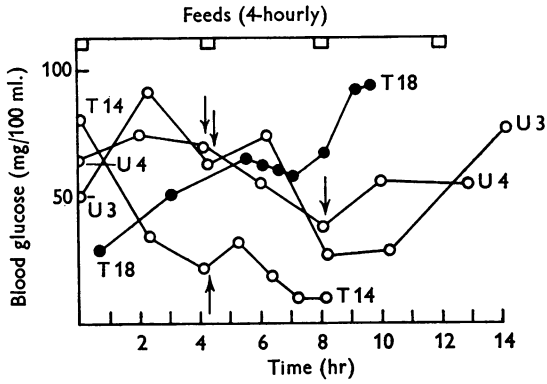
Text-fig. 1. The effect on the blood glucose concentration of 2 u. and 4 u. insulin/kg body wt. given intravenously to calves T 13 and G 6 respectively. The arrows indicate the time of injection.

The absorption of insulin

Oral administration in milk. After collecting basal blood samples for 4 hr, three calves (T 14, U 3, U 4), receiving milk at 4-hourly intervals, were given insulin 500 u./kg in the second feed; U 4 was given an additional identical dose in the third feed. The effects on the blood glucose level of the calves varied (Text-fig. 2). Two calves, U 3 and T 14 showed a slight but transient rise in blood glucose after the insulin feed. This rise was not observed in calf U 4 (Text-fig. 2). All these three calves subsequently showed a fall in blood glucose levels which may be contrasted with the generally rising levels shown by calf T 18 fed with milk only. The blood glucose values for T 14, which were decreasing even before insulin was given, had already reached 20·5 mg/100 ml. when the insulin was administered and subsequently fell to 9 mg/100 ml. Only this calf became distressed and a glucose solution (50 g/100 ml.) was therefore given both intravenously and subcutaneously after the last blood sample had been taken. Recovery was rapid and 3 hr later the calf was given a normal feed of pooled milk. Four hours later this calf was found in a coma. Further

glucose was administered and the calf recovered rapidly and permanently. Since this follows almost exactly the course of events described by Goodwin (1957*a*) after administering insulin subcutaneously to a calf it seemed that calf T 14 had absorbed some insulin. This was confirmed by the demonstration of insulin in the urine (see below, p. 212). The second insulin feed given to calf U 4, when it was 12 hr old, did not result in any further lowering of blood glucose. In fact, the values increased slightly during the ensuing 2 hr.

These results show first, that biologically active insulin can be absorbed from the intestine of the newly born calf and, when the initial level of



Text-fig. 2. The effects on the blood glucose concentration of 500 u. insulin/kg body wt. given orally in milk. T 14, U 3 and U 4 received insulin at the times indicated by the arrows. T 18 was given milk only (●——●). The volume of all feeds shown in Text-figs. 2–5 was 20 ml./kg body wt.

blood glucose is low, can induce a hypoglycaemic coma. Secondly, that insulin fed in milk is neither readily nor consistently absorbed from the intestine.

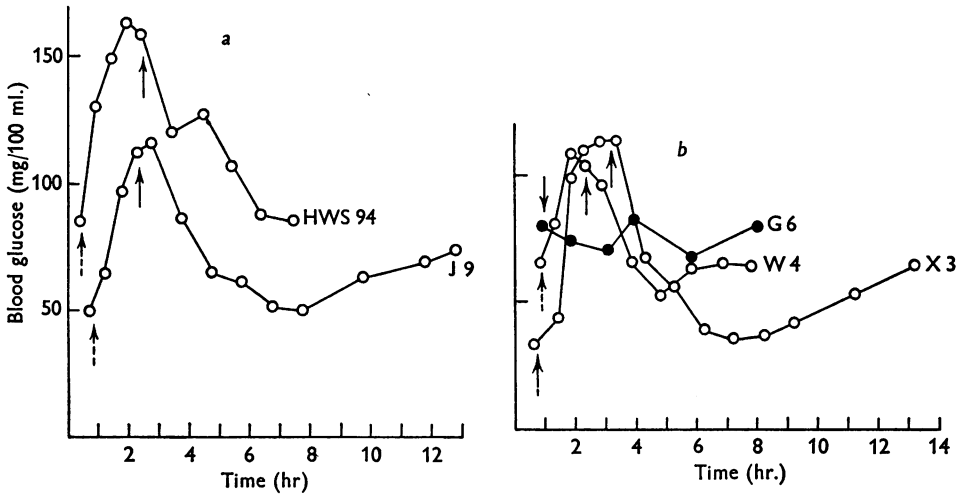
Oral administration of insulin in acid and alkaline solutions

In all but one of these experiments 5 % glucose, in the solvent to be used in the subsequent insulin feed, was given 1½–2 hr before the insulin. This was done to avoid low glucose levels similar to those observed in T 14 (see above). A better comparison could also be made between the changes, with time, in the blood glucose levels in the different animals. The insulin, 500 or 30 u./kg, was given by mouth in either saline (8·5 g/l., pH 4) or sodium bicarbonate solution (50 g/l., pH 7·9).

Preliminary experiments were carried out on two calves (HWS 94, J 9) to examine the changes in the blood glucose levels in the absence of insulin, that is, after a first feed of glucose-saline, followed 4 hr later by a second feed of saline alone. Two further calves (W 4, X 3) received

sodium bicarbonate-glucose followed by sodium bicarbonate alone. The results of these experiments are shown in Text-fig. 3*a* and *b*; the general rise in the blood glucose concentration curves for all four calves was similar. Where glucose determinations were carried out beyond the 7–8th hour, a steady rise in blood glucose was shown until the 13th hour; no glucose levels below 33 mg/100 ml. were recorded.

One calf (G 6) was given a feed of sodium bicarbonate solution alone shortly after birth. There was no effect on the blood glucose level, which was maintained at a mean value of 76 mg/100 ml. for the next 7 hr (Text-fig. 3*b*).



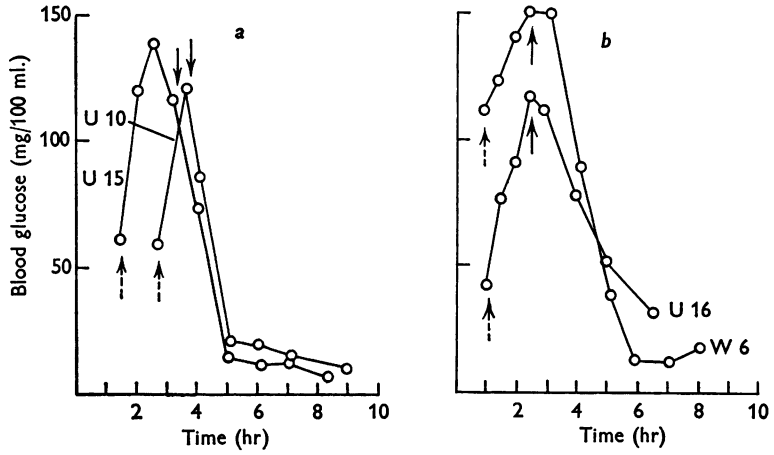
Text-fig. 3. The effect on blood glucose concentration of *a* Glucose (1 g/kg body wt.) in sodium chloride solution, 0·85 g/100 ml. ↑ Glucose-saline feed, ↑ feed of saline only. *b* Glucose (1 g/kg body wt.) in sodium bicarbonate solution, 5 g/100 ml. ↑ Glucose-bicarbonate feed, ↑ feed of bicarbonate only (Calf G 6 was given bicarbonate only ●——●).

Insulin 500 *u./kg body weight.* Two calves (U 10, U 15), given insulin in bicarbonate solution, after a previous feed containing 5% glucose in bicarbonate, showed a fall in blood glucose to values less than 20 mg/100 ml. within 1½ hr (Text-fig. 4*a*). Both calves became depressed, with laboured respiration. The low blood glucose levels were maintained for 3–4 hr when the experiments were terminated.

Two calves (W 6, U 16), given insulin in acid saline, did not show such a rapid hypoglycaemic response. The blood glucose concentration of calf W 6 fell to a value of 12 mg/100 ml. in 3 hr, but had begun to rise again before the end of the experiment (Text-fig. 4*b*). The blood glucose concentration of calf U 16 fell even more slowly. When the experiment was terminated, 4 hr after the insulin had been given, the blood glucose value

of U 16 was 31 mg/100 ml. (Text-fig. 4*b*). Both these calves appeared normal throughout the experiments.

Insulin 30 u./kg body weight. At this lower insulin dosage a clear difference was seen between calves (H 5, U 12) which received insulin in bicarbonate solution (Text-fig. 5*a*) and other calves (W 5, X 5, HWS 63) which were given insulin in acid solution (Text-fig. 5*b*). The changes in blood glucose concentration of calves H 5 and U 12 were very similar (Text-fig. 5*a*). A hyperglycaemic peak, due to the bicarbonate-glucose feed, was reached at approximately the same time. After the insulin feed both showed a rapid fall to about 35 mg/100 ml. followed by a more



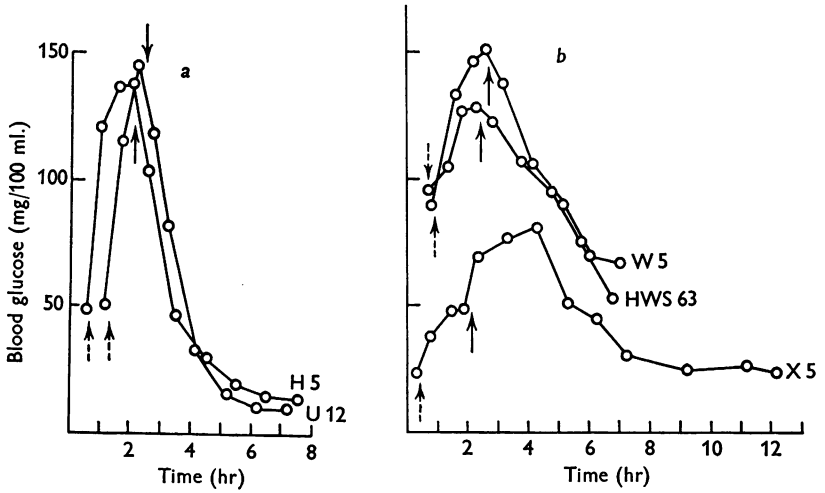
Text-fig. 4. The effect on blood glucose concentration of *a* Insulin (500 u./kg body wt.) given orally in sodium bicarbonate solution (5 g/100 ml., pH 7·9). ↑ Glucose-bicarbonate feed, ↑ insulin in bicarbonate. *b* Insulin (500 u./kg body wt.) given orally in sodium chloride solution (0·85 g/100 ml. pH 4·0). ↑ Glucose-saline feed, ↑ insulin in saline.

gradual fall to about 12 mg/100 ml. Both calves were given glucose at the end of the experiment, although only H 5 had shown any visible signs of hypoglycaemia.

Calves W 5 and HWS 63, which received the insulin at acid pH, did not show the same rapid fall in blood glucose concentration nor were the same low values reached (Text-fig. 5*b*). Following the glucose feed, the blood glucose concentration rose to a level of 130–150 mg/100 ml. (similar to calves H 5 and U 12), but at the end of the experiment the blood glucose concentration for both W 5 and HWS 63 was still greater than 50 mg/100 ml. The third calf (X 5, Text-fig. 5*b*) was examined over a longer period. This calf had a low basal blood glucose concentration (23 mg/100 ml.), which was not elevated very much during the 1½ hr following the

first glucose feed. During the first 2 hr after the second feed, containing insulin in acid saline, the blood glucose curve rose further to a peak value of 80 mg/100 ml. Blood glucose concentration then decreased during the next 3 hr and a level of about 25 mg/100 ml. was established.

The results indicate that insulin, fed in a bicarbonate solution, was more readily absorbed than an acid solution of insulin. Under these conditions a significant lowering of blood glucose levels was still shown when the dose of insulin fed was reduced from 500 to 30 u. insulin/kg body weight.



Text-fig. 5. The effect on blood glucose concentration of *a* Insulin (30 u./kg body wt.) given orally in sodium bicarbonate solution (5 g/100 ml. pH 7·9). ↑ Glucose-bicarbonate feed, ↑ insulin in bicarbonate. *b* Insulin (30 u./kg body wt.) given orally in sodium chloride solution (0·85 g/100 ml. pH 4·0). ↑ Glucose-saline feed, ↑ insulin in saline.

The pH of gastro-intestinal contents

Two calves were killed 4 hr after they had received 500 u. insulin/kg in sodium bicarbonate solution and acid saline respectively. After ligation, lengths of the gastro-intestinal tract were removed and washed out separately. The small intestine was divided into four approximately equal lengths. After filtration, the pH values of the contents together with the washings from each length of intestine were determined by means of a Pye 'Dynacap' pH meter. The values obtained are given in Table 1. Portions of the filtrates of all the samples, except those derived from the large intestine, were examined further. The addition of N-HCl resulted in the formation of precipitates. The addition of small amounts of acid insulin solution (pH 3·4) also produced precipitates which redissolved on shaking, except those formed with samples obtained from the stomach complex and the proximal section of the small intestine of the calf fed with insulin

in saline. There was no precipitation on adding alkaline solution of insulin (pH 8) to any of the samples.

TABLE 1. The pH values of the gastro-intestinal contents of calves which were given insulin (500 u./kg) in 5% sodium bicarbonate solution or in acid saline

Sample		Sodium bicarbonate (pH)	Acid saline (pH)
Solution fed		7·9	4·0
Stomach complex		7·82	5·55
Small intestine	section 1	7·05	6·22
	section 2	7·14	6·48
	section 3	7·12	6·73
	section 4	7·11	6·73
Large intestine		6·80	6·64

Serum insulin levels

Experiments were carried out which correlated the hypoglycaemia, which had occurred in some of these calves, with an increase in the level of insulin-like activity in the blood. Serum samples were obtained from two calves (U 15 and X 5).

Two samples were obtained from calf U 15. The first was taken 1½ hr after glucose had been given, at which time the blood glucose concentration was at its highest value. The calf then received a feed containing 500 u. insulin/kg in sodium bicarbonate solution, and the second sample was obtained 5 hr later when the blood glucose concentration was extremely low. Samples were taken from the second calf at 0, 1½ and 12 hr. Glucose was given immediately after the first sample had been obtained and insulin (30 u./kg; saline pH 4·0) after the second sample. This insulin had no appreciable hypoglycaemic effect on the calf (Text-fig. 5*b*). The results, given in Table 2, indicate that the glucose caused a rise in blood insulin level but that the rise in activity after the oral dose of insulin, which produced profound hypoglycaemia in calf U 15, was considerably greater. The values of 210 and 160 μu./ml., obtained before and after the hyperglycaemic peak produced in calf X 5, are comparable with those found for normal cow and calf serum by Cunningham (1962) (100–500 μu./ml.), using a rat diaphragm assay to estimate insulin activity in acid-ethanol extracts of plasma.

Route of transport

The route of insulin transport was examined in an anaesthetized calf by determining the insulin-like activity in samples of lymph (thoracic duct) and blood (jugular vein) obtained after giving insulin (500 u./kg) in sodium bicarbonate intraduodenally (see Methods, p. 204). The insulin-like activity was determined by immuno-assay. Following the cannulation of the thoracic duct, a surgical attempt was made to eliminate all collateral

TABLE 2. Blood glucose concentration and serum insulin-like activity (S.I.L.A.)

	Time (hr min)	Details of feeds at 20 ml./kg body wt.	Blood glucose (mg/100 ml.)	S.I.L.A.* (μu./ml.)
Calf X 5	0 15	—	23	210 (130–370)
	0 40	5 % glucose in 0·85 % NaCl, pH 4·0		—
	0 45	—	38	—
	1 25	—	48	—
	1 50	—	48	1000 (500–13,000)
	2 05	30 u.insulin/kg in 0·85 % NaCl, pH 4·0		
	2 15	—	70	—
	3 15	—	76	—
	4 15	—	81	—
	5 15	—	61	—
	6 15	—	45	—
	8 15	—	31	—
	10 15	—	25	—
	11 15	—	27	—
	12 15	—	24	160 (80–270)
Calf U 15	0 25	—	60	—
	0 30	5 % glucose in 5 % $NaHCO_3$, pH 7·9		
	1 05	—	118	—
	1 35	—	137	900 (300–2700)
	2 15	—	115	—
	2 30	500 u.insulin/kg in 5 % $NaHCO_3$, pH 7·9		
	3 00	—	72	—
	4 00	—	13	—
	5 05	—	11	—
	6 00	—	12	—
	7 20	—	6	(47,300 (36,600–61,200)

* Estimated from measurements of glucose uptake by rat adipose tissue, 95 % confidence limits given in parentheses.

lymphatic vessels. However, it was possible that some visceral lymph may still have reached the general circulation.

Three samples of lymph were examined. Each sample consisted of all the lymph collected over a period of approximately 30 min. Blood samples were taken at intervals during the experiment. The lymph and the blood samples obtained before insulin was given contained less than 1 mu./ml. insulin-like activity. After administration of insulin through the cannula placed in the duodenum the insulin-like activity rose in both lymph and blood. The rise in activity was greatest in the blood. A blood sample taken 35 min after the insulin was given contained 43 mu./ml., whereas lymph collected between 30 and 50 min after the insulin was given contained only 16 mu./ml. These results suggest that most of the absorbed insulin passed directly into the blood.

Insulin in urine

Urine was collected from calves U 15 and T 14 before insulin was administered and again at times after giving 500 u. insulin/kg body weight in 5 % sodium bicarbonate solution and milk respectively. U 15 had

received a feed of glucose in sodium bicarbonate solution and T 14 a feed of milk before any urine was collected. The urine samples were examined immunologically by double diffusion in agar gel against an antiserum to bovine insulin prepared in the guinea-pig. No reaction was obtained with the urine collected before insulin was given (Pl. 1). The first two samples obtained from calf U 15 after insulin administration gave negative results, but the next sample, which was obtained $5\frac{1}{2}$ hr after the insulin was given, gave a precipitin line. When this sample was collected, the blood glucose concentration was 6 mg/100 ml. and had been less than 15 mg/100 ml. since the previous urine sample was obtained. The first two samples of urine collected from T 14 after insulin had been given also gave a precipitin reaction with the antiserum. The calf then became comatose and was therefore given glucose. The next urine sample collected 3 hr later did not give a precipitin reaction with the antiserum.

Since the urine samples were of bovine origin, the specificity of the immunological reaction was examined. Adult and calf bovine serum, bovine milk and a protein fraction from bovine pancreas, from which insulin had been exhaustively extracted, gave no precipitin reaction with the antisera, as has been reported previously (Birkinshaw *et al.* 1962).

The results obtained by the immunological technique on the urine samples from calf U 15 were confirmed by mouse convulsion assays. No activity was detected in the samples which were negative in the double diffusion test. The sample which gave a precipitin line contained 4·5 u./100 ml. The assay technique would have detected 0·1 u./100 ml. in any of the samples.

In a control experiment (calf W 4) urine was collected for 7 hr after administration of glucose in sodium bicarbonate solution. None of the four samples contained detectable amounts of insulin. During the time of the experiment the blood glucose concentration was elevated, following the glucose dose, and had been restored to the pre-feeding level (Text-fig. 3*b*). The secretion of endogenous insulin in response to a glucose load did not therefore result in the appearance of insulin in the urine.

DISCUSSION

Goodwin (1957*b*) showed that blood glucose values varied considerably from calf to calf immediately after birth and this was confirmed in the present experiments. For this reason, in the later experiments insulin was given $1\frac{1}{2}$–2 hr after a feed of glucose (1 g/kg body wt.). In this way the calves received the insulin at or about the time of the hyperglycaemic peak observed in control animals which received glucose but no insulin. The effect of insulin absorption is shown by an increase in the slope of the descending limb of the blood glucose curve and the lower blood glucose

evels reached, compared with the control animals. This is therefore similar to the modified glucose-insulin tolerance test of Lazarus & Volk (1952).

The results show that insulin, administered orally, was absorbed and passed into the general circulation of the newly born calf. Insulin appeared to be more readily absorbed when given in an alkaline rather than acid solution. This may be related to the solubility of insulin. The minimum solubility for insulin in aqueous solution occurs at the iso-electric point of pH 5·2. However, there is some alteration of this value in the presence of other solutes so that a precise value cannot be given for a particular physiological circumstance.

Gastric acid secretion in the newly born animals of certain species, including the calf, is considerably less than in the adult (Hill, 1956; Pierce, 1962). Therefore it is not surprising that the pH remained alkaline in the stomach contents of those calves fed with insulin in alkaline solution (see Table 1). Under these circumstances insulin, fed in solution on the alkaline side of the iso-electric point, would not be precipitated in the stomach nor subsequently in the small intestine. On the other hand, insulin fed in saline at pH 4·0 would remain soluble in the stomach, but when it subsequently passed into the small intestine the pH would be raised through the iso-electric point (see Table 1). Thus it is probable that insulin would come out of solution in the proximal part of the intestinal lumen and may or may not re-dissolve, depending on the conditions existing in the gut at that particular time.

Insulin in acid solution, fed by mouth at 1 u. and 0·4 u./g body wt., effectively lowered the blood glucose of newly born mice (Kelly, 1960) and rats (Mosinger *et al.* 1959) respectively. In the present studies 0·03 u./g body wt. was effective when given in alkaline solution. However, there is evidence that the intestinal mucosa of newly born rats shows a selective preference for the absorption of certain homologous rather than heterologous proteins (Halliday, 1955, 1957). For the calf the insulin was homologous, whereas for the rat and mouse it was heterologous. This may be an additional or alternative explanation for the differences in effective dose levels of bovine insulin in the newly born of different species.

In one new-born calf it was possible to examine the route of transport of insulin absorbed from the gastro-intestinal tract. The result of this experiment is in agreement with those obtained with serum albumin (Balfour & Comline, 1959), showing that low-molecular-weight proteins can enter the blood directly as well as via the lymph. Moreover, the result suggests that more of the insulin was transported in the blood than in the lymph.

The detection of insulin in urine confirms that low-molecular-weight

protein can be excreted intact by the kidney. This fact supports previous evidence, which showed that proteinuria in the new-born calf arises from low-molecular-weight protein absorbed from ingested colostrum (Pierce, 1959, 1961). However, it seems from these experiments that insulin can only be expected to appear in the urine when the level in the blood is raised to very high values. Since excretion of the hormone was associated with severe hypoglycaemia, it may only be possible to demonstrate excretion of insulin in the newly born animals of those species, such as the calf, which can tolerate extremely low blood glucose concentrations and still maintain their general body metabolism (Goodwin, 1957*b*).

SUMMARY

1. The absorption of orally administered bovine insulin has been examined in calves less than 24 hr old. Changes in the blood glucose level and in plasma insulin-like activity have been used as indicators of the absorption of insulin in a biologically active form.

2. In these experiments more insulin was absorbed from an alkaline solution (pH 8·0) than from saline at pH 4·0 or from milk (pH 6·7). The difference between the results obtained in these experiments has been discussed with reference to the changes in the solubility of insulin around its iso-electric point.

3. Insulin-like activity has also been measured in calf blood and lymph after oral and intra-duodenal administration of insulin. The results suggest that more insulin is transported from the intestine in the blood than in the lymph.

4. Insulin-like activity has been shown in the urine of calves made severely hypoglycaemic as the result of oral insulin.

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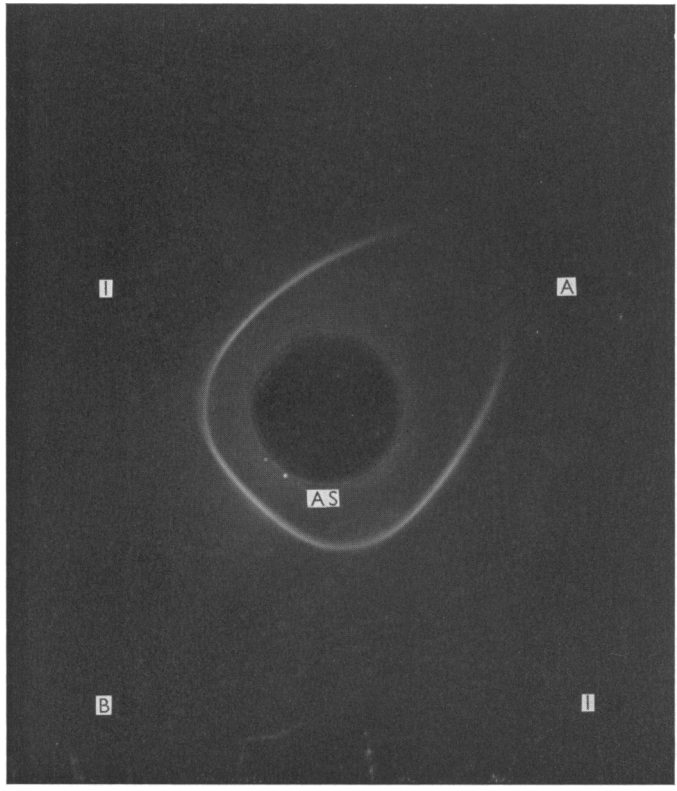
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EXPLANATION OF PLATE

Precipitin lines formed in agar gel by the double diffusion of guinea-pig anti-bovine insulin serum and concentrated (× 25) urine samples from a calf given insulin (500 u./kg body wt.) orally in sodium bicarbonate solution (5 g/100 ml. pH 7·9). *AS* Guinea-pig anti-insulin serum; *I* Insulin solution (1 u./ml.); *A* Urine collected before feeding insulin; *B* Urine collected 5½ hr after feeding insulin.