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ACCELERATION OF THE ABSORPTION OF UNCHANGED GLOBULIN IN THE NEW-BORN CALF BY FACTORS IN COLOSTRUM

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The transfer of maternal antibodies to the new-born animal through the colostrum was first noted by Ehrlich (1892), but the phenomenon does not appear to have been examined in detail until Howe (1921) defined its characteristic features in his study of the changes in serum proteins which follow the ingestion of colostrum in the calf. In his experiments he showed that in the new-born animal colostrum globulins were absorbed without change in amounts sufficiently large to alter substantially the composition of the blood plasma and that this type of absorption could only occur within a relatively short period after birth. The length of this period varies between species, but in many, including the ruminants, it is limited to the first 24–36 hr after birth.

The principal protein which appears in the blood serum after colostrum administration is the immune lactoglobulin, and later work, in which the serum proteins have been compared with those of the colostrum whey by electrophoresis and by the use of the ultracentrifuge, has fully confirmed that there is no detectable change in the structure of the protein during its absorption and appearance in the blood serum of the new-born animal (Jameson, Alvarez-Tostado & Sortor, 1942; Johnson & Pierce, 1959). Further investigations have shown that the protein is absorbed through the small intestine and that the unchanged immune lactoglobulin is carried by the lymph to the peripheral blood and does not enter the portal blood vascular system in appreciable amounts (Comline, Roberts & Titchen, 1951*a*). In the calf the absorption is not specific to the colostrum globulins, and other proteins such as the bovine serum globulins, serum albumin and even polysaccharides with a high molecular weight such as dextran, can be absorbed during this time (Balfour & Comline, 1959*b*).

In the experiments of this paper the selective entry of γ-globulin into the lymphatic capillaries and thence into the thoracic or intestinal lymph ducts has been used to examine the rate of absorption of globulin under different conditions. The colostrum has been found to contain several

factors which accelerate the absorption of globulin from the small intestine during the relatively short period after birth when this type of absorption can occur. A change in these factors in the colostrum is not responsible for the waning of the absorption after 24 hr of life. A short summary of the experiments has been published previously (Balfour & Comline, 1959*a*).

METHODS

Animals. Newly born male Jersey calves which had not been allowed to feed in any way were used in these experiments. In order to ensure that they did not suckle colostrum, it was essential to separate the calves from their mothers immediately after birth. In the majority of experiments the preparation was complete and the infusion into the duodenum started from 3 to 17 hr after birth.

Dissection. Anaesthesia was induced and maintained with sodium pentobarbitone ('Nembutal'; Abbott Laboratories) injected into the jugular vein. Cannulae were inserted into the trachea and the femoral vein.

The thoracic duct was sought in its extra-pleural course in the triangle formed by the sternocephalic muscle and the jugular vein and by the retracted cleido-occipital and deep pectoral muscles. In many cases the duct was found near the inferior cervical artery: if possible it was cannulated at the ampulla close to the jugular vein. The duct was normally dissected free from the fascia in which it lay for a length of 2–3 cm in order to reduce the possibility of overlooking other branches in communication with the jugular or other veins. If any of these branches were found they were ligated; in these circumstances, unless the character and rate of flow of the lymph from the cannulated duct indicated that all other communicating branches had been tied off, the experiment was abandoned.

The main intestinal lymphatic duct was identified in many animals on the ventral face of the posterior vena cava and the left renal vein. Where possible it was cannulated close to the left kidney or near the left adrenal gland.

Cannulae were inserted into the duodenum close to the pylorus. Particular care had to be taken to avoid damage to the vessels of the duodenal mesentery during the insertion of the ligatures. A large-bore glass cannula was tied into the blind free end of the caecum. In the early experiments a ligature was also tied round the colon distal to the ileocaecal valve. Subsequent experiments, in which the course of the colostrum whey through the intestine was traced by the addition of Indian ink to it, showed that this ligature was unnecessary and that very little of the intestinal contents which passed through the ileocaecal valve entered the ascending colon; the major part was delivered from the caecal cannula.

Colostrum whey. Colostrum, freed from its fat, was clotted by incubation at 37° C with a commercial rennin ('cheese rennett'; Fullwood & Bland). Clear whey was collected as the casein clot contracted, and immediately cooled, filtered and stored at −20° C in 550 ml. portions. The rennin preparation used contains a high concentration of NaCl. Although only 1–2 ml. was used for each litre of colostrum, the addition of NaCl to the whey was avoided in later experiments by dialysis of the rennin followed by freeze-drying. This preparation retained its activity when stored in ampoules. In all experiments 500 ml. of colostrum whey or other solutions was delivered to the duodenum from a reservoir connected by tubing to a Murphy drip chamber, which was in turn connected to the duodenal cannula. The flow into the duodenum was regulated to 50 ml./15 min.

Lymph volumes were measured for successive periods of 10 min. Equivalent volumes of Krebs–Ringer bicarbonate, prepared from the formula given by Umbreit, Burris & Stauffer (1945), were infused into the circulation via the femoral venous cannula to replace the lymph.

Heparin (Roche: 250 i.u./kg) was injected intravenously shortly after the cannulation of the thoracic duct: it was sometimes necessary to repeat this dose after 100–120 min.

Throughout the experiment 5% CO_2 in oxygen was administered at a rate of 2–3 l./min through the tracheal cannula.

Preparation of ^{131}I-labelled globulin. ^{131}I-labelled globulin was prepared by the method described by Francis, Mulligan & Wormall (1954) and in later experiments as described for albumin by Veall (1954). This latter method has the advantage of speed, since it eliminates the need for repeated protein precipitation and subsequent dialysis.

Preparation of artificial colostrum. Bovine serum γ-globulin (Armour) was dissolved as 2 g or 4 g/100 ml. solution whose ionic composition was similar to that of ultrafiltrate of colostrum whey. When inorganic phosphate was omitted from the salt mixture, chloride was substituted so that the Na:K ratio was not disturbed. When required, the colostrum whey was ashed by the method of McCance, Widdowson & Shackleton (1936). The dibarium salt of glucose-6-phosphate was dissolved in dilute HCl and the Ba precipitated as $BaSO_4$ with Na_2SO_4. The resulting solution of the Na salt was neutralized before addition to the artificial media. Phosphorylcholine was prepared by the method of Plimmer & Burch (1937) and glycerophosphorylcholine by the method of Dawson (1956). The small protein fraction was prepared from fresh colostrum by the first stage of the method of Laskowski & Laskowski (1951) for the isolation of the colostrum trypsin inhibitor. The material resulting from the second precipitation with $(NH_4)_2SO_4$ was dissolved in water and extensively dialysed against distilled water. Thereafter it was either freeze-dried or stored as a solution at $-20°$ C. When required pepsin (British Drug Houses) was used at a concentration of 40 mg/100 ml.

Analysis and identification of the phosphate components in the ultrafiltrate of colostrum whey was carried out according to the barium and alcohol fractionation procedure described by Umbreit *et al.* (1945). The phosphate esters found in the barium-soluble, alcohol-insoluble, fraction were studied by paper chromatography, using the method described by Hanes & Isherwood (1949) and the solvents of Gerlach, Weber & Döring (1955). Phosphate analysis was conducted by the Berenblum & Chain (1938) micromethod. Zone electrophoresis of the ^{131}I-labelled γ-globulin in the lymph was carried out in a cellulose column with phosphate-borate buffer, ionic strength 0·05, pH 8·6, according to the instructions of Gedin & Porath (1957).

Measurement of the radioactivity in the lymph. The cannula from either the thoracic duct or the main intestinal duct was connected by polythene tubing to a liquid flow Geiger-Müller counter (F.W. 10, 20th Century Electronics) which was coupled to a Panax model 100 c scaler. The radioactivity was determined for consecutive 10 min periods on the lymph flowing through the counter. The mean count rate per minute for each 10 min period was expressed as a percentage of the count rate per minute of the colostrum or solution which had been introduced into the duodenum.

RESULTS

The appearance in the lymph of bovine serum γ-globulin labelled with ^{131}I was used in the following experiments to study the rate and extent of the absorption of unchanged γ-globulin from the small intestine of the newly born calf. This source of globulin was chosen in preference to colostrum globulin, which requires preliminary separation from the many other proteins of the colostrum whey before labelling with ^{131}I. The use of ^{131}I-tagged protein had the added advantage that the process of absorption could be followed throughout the experiment. The technique, however, depended on the stability of the labelling of the protein with ^{131}I and the entry into the lymphatic rather than the blood-vascular capillaries of the major part of the γ-globulin after absorption.

Stability of the ^{131}I-labelled globulin

The possibility of the transfer of the ^{131}I from the γ-globulin to another protein fraction or the hydrolysis of the γ-globulin into smaller fragments during its absorption was checked in the following manner. Thoracic-duct lymph with the greatest amount of radioactivity was placed in a cellophane sac and dialysed against phosphate-borate buffer pH 8·6 at 5° C for 24 hr. No change in the radioactivity was detected in a sample when tested on a scintillation counter after corrections had been made for dilution and radioactive decay. Ten millilitres of the dialysed lymph was then subjected to zone electrophoresis for 42 hr in a 120 cm long ethanolized cellulose column. The separated proteins were then eluted and collected in 5 ml. fractions; 92 % of the radioactivity applied to the column was recovered in the most-slowly-moving fraction of the protein, which is characteristic of bovine serum γ-globulin.

Route of absorption of globulin

When ^{131}I-labelled bovine γ-globulin was absorbed by the small intestine of the newly born calf, the radioactivity could be readily detected in the lymph from either the thoracic duct or alternatively the main intestinal lymph duct. In contrast, only very small amounts of radioactivity were found in the blood taken from the jugular vein and its appearance was always delayed (Fig. 1).

From the results of the above experiments it was concluded that under those conditions the measurement of the radioactivity of the lymph provided an accurate and sensitive method for the study of the absorption of γ-globulin from the small intestine of the newly born calf.

Variation in the absorption of ^{131}I globulin in different wheys

The rate and speed of the absorption of ^{131}I bovine γ-globulin, as seen by the appearance of radioactivity in the lymph, was similar to that previously described for colostrum immune globulin (Comline, Roberts & Titchen, 1951*a*). Characteristically 60–120 min elapsed before any labelled protein could be detected; its first appearance was followed by an abrupt rise in the concentration over the next 20–30 min (Fig. 2). The maximum concentration of γ-globulin was found about 180–200 min after the start of the introduction of the whey into the duodenum. This concentration was then either maintained or fell slowly until the end of the experiment at or about 300 min.

Wide variations occurred in the rate at which bovine γ-globulin appeared in the lymph when it was dissolved in different samples of colostrum whey, although in all experiments the pattern of absorption retained its charac-

teristic sequence. These differences were also evident in the proportion of the dose initially dripped into the duodenum, which was recovered from the lymph during the experiment (see Fig. 2*a*). The much smaller absorption of protein after 24–36 hr first reported by Howe (1921) is shown in Fig. 2(*b*). In view of these results factors in colostrum whey which could influence the absorption of γ-globulin by the small intestine were sought.

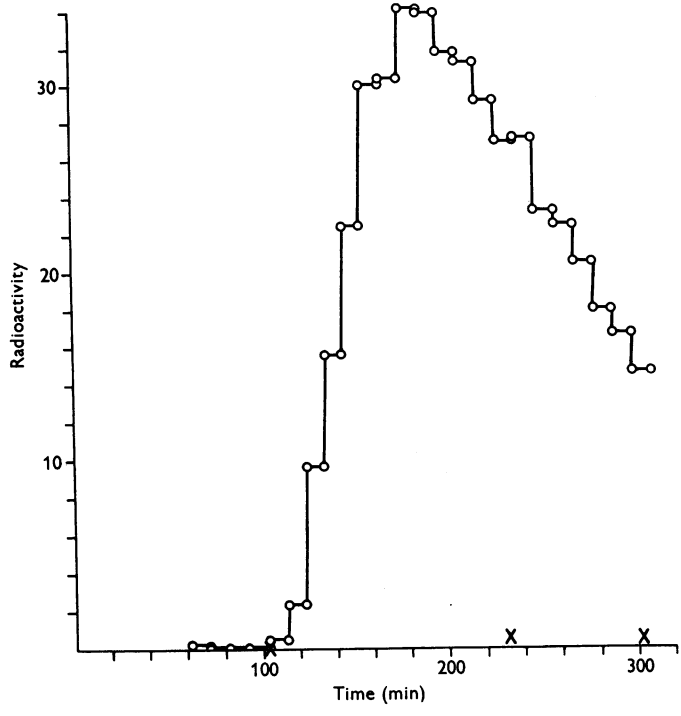

Fig. 1. Comparison of the radioactivity of the thoracic duct lymph (O—O) and the blood from the jugular vein (×) during the absorption of ^{131}I-labelled bovine serum γ-globulin dissolved in 500 ml. colostrum whey, dripped into the duodenum at the rate of 50 ml./15 min for the first 150 min. Age of calf, 4½ hr. In all Figs. except Fig. 11, ordinate shows radioactivity of lymph and blood expressed as percentage of count rate/min of the colostrum whey (see Methods); abscissa, time (min).

Identification of factors in colostrum whey which influence the absorption of globulin

The following experiments demonstrated that colostrum whey contains substances which substantially accelerate the absorption of globulin by the new-born calf. In Fig. 3 the rate and extent of the appearance of ^{131}I-labelled bovine serum γ-globulin in the lymph is compared after it was

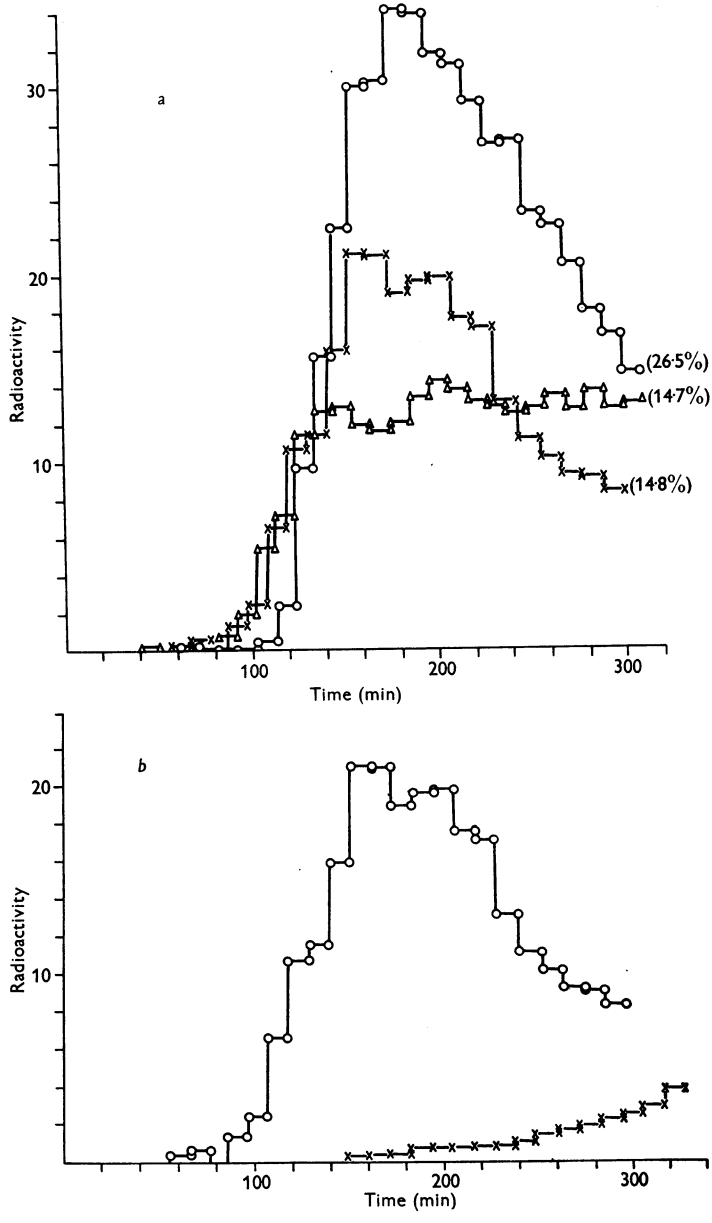

Fig. 2. (*a*) Variations in the rate of appearance of ^{131}I bovine serum globulin in the lymph when it was dissolved in wheys prepared from different samples of colostrum. Figures in brackets indicate percentage of the initial dose of ^{131}I globulin dripped into the duodenum recovered in the lymph during 300 min of experiment.

(*b*) The decrease in the absorption of globulin after 24 hr of age. Identical samples of colostrum whey with added ^{131}I serum globulin were used in calves at the following ages: O—O, at 10½ hr; ×—×, at 40½ hr.

administered either in colostrum whey or in a 2% solution of serum γ-globulin dissolved in the following solutions:

(1) Colostrum whey from which the heat-coagulable protein had been removed; to do this the whey was adjusted to pH 5·4 and boiled for 5 min. It was then cooled and filtered, adjusted to pH 6·8, and 2% bovine serum γ-globulin dissolved in the clear filtrate.

(2) Solutions with similar concentrations of Na, K, Ca, Mg to those of colostrum whey, prepared from the chloride salts of these cations; the concentrations of these cations in colostrum whey or the filtrates derived from them were found to lie within the following values (mM): Na 45–76, K 40–60, Ca 20–30, Mg 9–11.

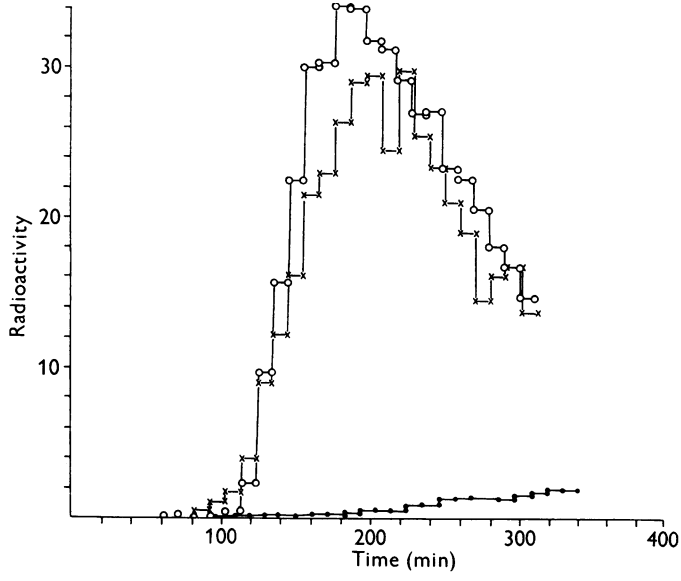

Fig. 3. Comparison of the rate of appearance of ^{131}I globulin in the lymph when it was given either in fresh colostrum whey (○—○), or in a 2% solution of bovine serum γ-globulin dissolved in (i) the clear filtrate derived from this whey after removal of the heat-coagulable protein (×—×) or (ii) a chloride solution with the same Na, K, Ca, Mg as in whey (●—●).

In the solutions used in the experiments the concentrations were (mM): Na 56·7, K 44·8, Cl 101·5.

It is evident from Fig. 3 that the clear filtrate obtained from colostrum whey after removal of the heat-coagulable protein retained the factors which accelerate globulin absorption and that the pattern of absorption from the filtrate was very similar to that from the colostrum whey from which it was prepared. The filtrates showed the same individual variations in their effects upon absorption as the colostrum wheys and this was reflected in

the proportion of the ^{131}I-labelled globulin originally dripped into the duodenum recovered from the lymph during the 300 min of the experiments; this varied between 10 and 30 %.

In contrast, absorption from the solution of chloride salts was very slow, the typical rise in the radioactivity of the lymph between 60 and 120 min was absent and even after 300 min it was only just detectable. In consequence only about 0·9–1 % of the material introduced into the duodenum was recovered from the lymph. The absorption was as low if not lower when the serum γ-globulin was dissolved in 0·9 % NaCl. The addition of 2 % (w/v) lactose or glucose to the chloride solution did not affect the absorption of globulin from these solutions.

The clear filtrate from colostrum whey retained the property of acceleration of globulin absorption for several weeks if it was stored at $-20°$ C. It provided, therefore, a convenient source of material for the further analysis of the factors responsible for this acceleration. In all the experiments described below the addition of 100 mg of globulin labelled with ^{131}I to solutions containing 2 or 4 g/100 ml. of bovine serum γ-globulin allowed the effect of different substances on the absorption to be followed from the radioactivity of the lymph.

The following experiments were carried out to ascertain the properties of the active substances present in the filtrates of colostrum whey.

Treatment with acid and alkali. The activity of the filtrates of colostrum whey was tested after they had been boiled under a reflux condenser for 1 hr with either HCl or NaOH which were added to give a final concentration of 0·1 N. In each case the treated extracts were adjusted to pH 6·8 before the addition of 2 % globulin and its introduction into the duodenum. Figure 4 shows that the activity remained after such treatment with HCl and was still present, although its effects were reduced after boiling with NaOH.

The addition of NaCl to the heat-treated colostrum whey reduced the amount of globulin absorbed into the lymph. In Fig. 5 comparison is made between the absorption from fresh colostrum whey and a similar sample of whey to which NaCl 100 mM had been added: in other experiments where there was a high concentration of NaCl in the globulin solution the absorption was similarly very small. In these cases it proved possible at the end of the experiment to collect a large fraction of the administered globulin solution. Determination of the radioactivity of these samples showed that between 40 and 95 % of that administered could be recovered from the caecum. With solutions whose cation composition was similar to that found in the whey attempts were made to recover the lumen contents but the volume yielded was too small to make the calculation of recovery of unabsorbed protein worth while.

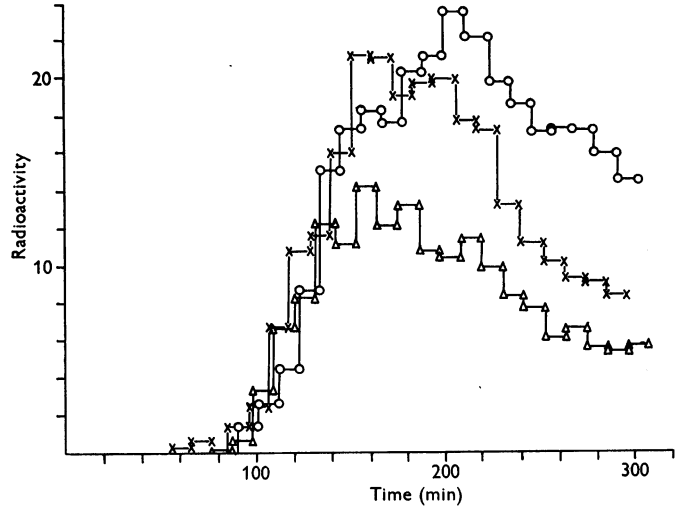

Fig. 4. Absorption of bovine serum γ-globulin when dissolved as a 4% (w/v) solution in heat-treated whey after the following treatments: O—O, after boiling with 0·1 N-HCl for 60 min and subsequent adjustment to pH 6·8; △—△, after similar treatment of heat-treated whey with 0·1 N-NaOH; ×—×, absorption from the original colostrum whey.

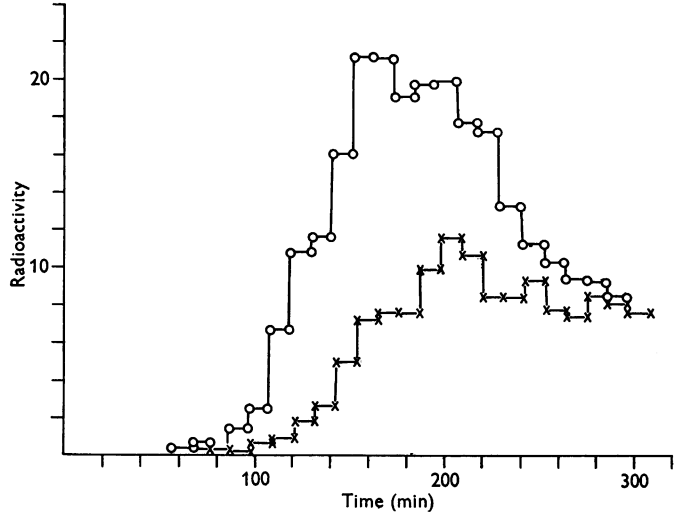

Fig. 5. Effect on the absorption of serum γ-globulin of the addition of NaCl to colostrum whey. O—O, absorption during control experiment with colostrum whey; ×—×, absorption after addition of NaCl (100 mM) to the colostrum whey.

Removal of other proteins not coagulated by heat. The filtrate recovered from the colostrum whey after removal of the proteins coagulated by heat still contained a considerable quantity of protein. This residual protein could be removed either by precipitation with trichloroacetic acid or by digestion with pepsin.

Effect of trichloroacetic acid precipitation. Trichloroacetic acid (5 g/100 ml.) was added to the filtrate; the further addition of trichloroacetic acid produced no more precipitation of protein. The traces of trichloroacetic acid were then removed from the filtrate by several extractions with ether and the ether was removed by aeration. This filtrate was used as a solvent for the bovine serum γ-globulin.

The onset of the absorption of bovine serum γ-globulin was delayed when it was administered in filtrates freed from trichloroacetic-acid precipitable material. Furthermore, high levels of radioactivity in the lymph were only encountered towards the end of these experiments (Fig. 6 *a*). The pattern of absorption, in fact, resembled that found with solvents which contained inorganic phosphate and glucose-6-phosphate, described in a later section (see Fig. 8).

Effect of digestion with pepsin. The heat-stable protein in solution was also removed from the filtrate by incubation with pepsin 40 mg/100 ml. at pH 2·0. After digestion for 5 hr at 37° C the solution no longer gave a precipitate when tested with trichloroacetic acid (5 g/100 ml.). The influence on the absorption of bovine serum γ-globulin was similar to that found after trichloroacetic-acid precipitation, in that the absorption was delayed both in time of onset and in achieving the maximum concentration in the lymph (Fig. 6 *b*).

These experiments, therefore, provided evidence that a protein which was not coagulated or inactivated by heat appeared to be important in accounting for part, at least, of the accelerating action of colostrum on globulin absorption from the small intestine of the new-born calf. This conclusion was supported by the effects of dialysis of the filtrates derived from the colostrum whey.

Effect of dialysis was tested in the following manner. Five hundred millilitres of filtrate from colostrum whey was dialysed in a cellophane sac for 20–24 hr against 1 l. of distilled water. The dialysate was evaporated under reduced pressure to 500 ml. and used as a solvent for 2 % globulin. The non-dialysable material retained within the cellophane sac was dialysed for a further 72 hr against further changes of distilled water: it was then recovered and NaCl and KCl added to give concentrations of Na and K similar to that in the original filtrate (mM: Na 57, K 45). The results of these experiments are shown in Fig. 7. It is clear that the major part of the activity was recovered from the dialysate; but again, as in the

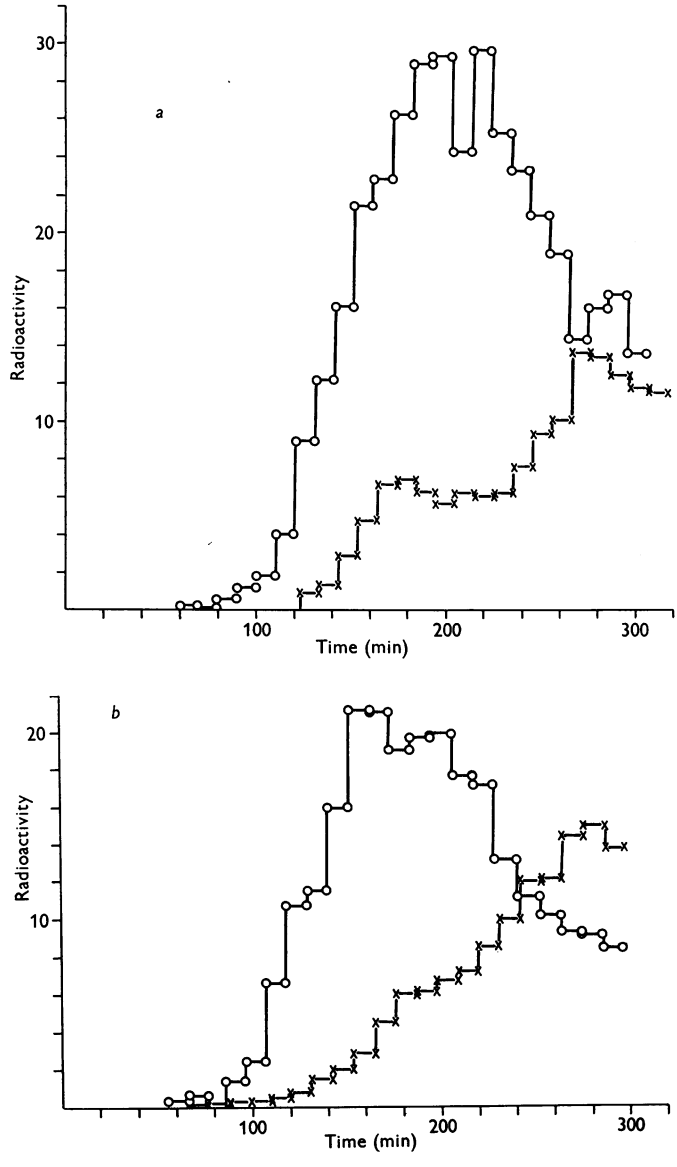

Fig. 6. Effect of the removal of proteins which remain after heat-coagulation of colostrum whey, on the absorption of γ-globulin: (*a*) after precipitation of these proteins with trichloroacetic acid; (*b*) after their digestion with pepsin. O—O, control experiment with whey filtrate; ×—×, after removal of proteins not coagulated by heat from the filtrates.

experiments after pepsin digestion, the pattern of absorption was shifted to the right when compared with that of the original colostrum filtrate. On the other hand only slight residual acceleration of globulin absorption occurred after thorough dialysis of the soluble protein of the whey extracts.

The results suggested that, in addition to a protein factor in colostrum whey which accelerated γ-globulin absorption, there were factors which both resisted peptic digestion and passed through cellophane membranes and which also accelerated globulin absorption through the intestine. Attempts to identify these factors are described below.

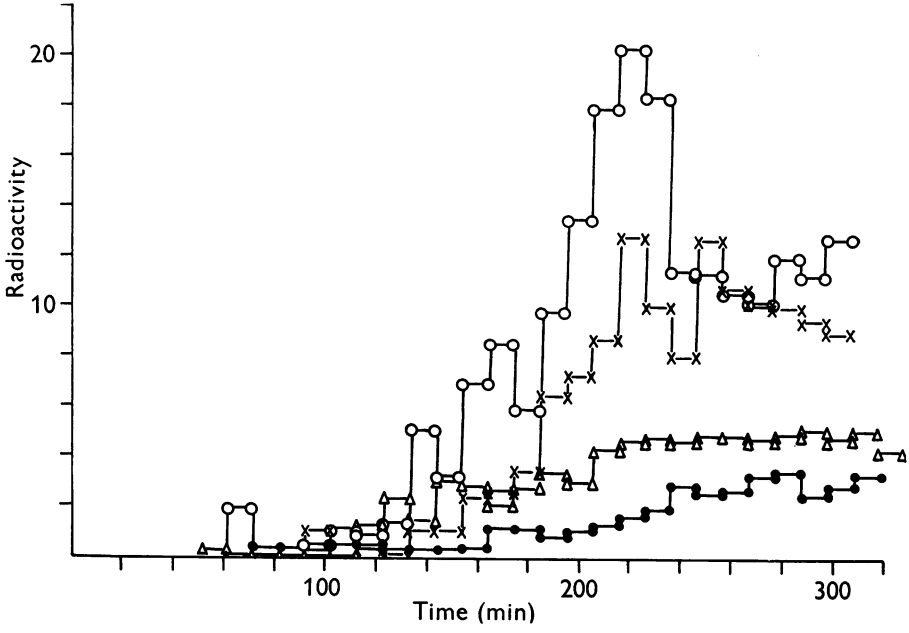

Fig. 7. Effect of dialysis upon the activity of filtrates from heat-treated colostrum whey. ×—×, ○—○, absorption from dialysate; △—△, ●—●, absorption from a solution of non-dialysable material.

Recombination of factors present in colostrum whey

Inorganic phosphate. The experiments described above had shown that only traces of bovine serum γ-globulin could be identified in the lymph if it was administered in a solution in which the Na, K, Ca and Mg concentrations were similar to that of the colostrum whey or the filtrate derived from it (Fig. 3). In all these experiments, however, the cations were added as the chlorides, the concentration of which in colostrum whey is not high (20–30 mM). Similarly a solution obtained after the complete incineration of the colostrum whey, followed by leaching of the ash with HCl and subsequent adjustment to pH 6·8, had no effect on the rate of absorption.

This solution, however, contained a high concentration of NaCl, which had been shown to depress absorption (Fig. 5) and did not contain all the inorganic components of the whey, particularly phosphate.

In further experiments part of the chloride was replaced by inorganic phosphate so that the concentration of the solution dripped into the duodenum in respect of both these anions was similar to that of colostrum whey; to do this inorganic phosphate 20 mM was substituted for chloride, the concentration of which was reduced from 101·5 to 81·5 mM; the Na and K concentrations remained at 56·7 and 44·8 mM, respectively.

The addition of inorganic phosphate accelerated the absorption of serum globulin (Fig. 8*a*). The effect was not, however, comparable to that of whey or the filtrate derived from it. The rise in the radioactivity of the lymph occurred more slowly and was often delayed until after 300 min from the start of the experiment. Thus the abrupt rise between 60 and 120 min so characteristic of colostrum whey was absent, and the effect of inorganic phosphate resembled but was slower than the absorption found after removal of the heat-stable proteins from the filtrates of whey. The phosphate effect in different animals was also less regular, as is reflected in the variations in the total amount of globulin recovered from the lymph in 300 min in the different experiments.

Glucose-6-phosphate. The differences in the rates of absorption found with inorganic phosphate and colostrum whey from which the heat-stable proteins had been removed led to a search for a more active phosphate ester. Phosphorylcholine and glycerophosphorylcholine and choline itself, at a concentration of 7·2 mM, were inactive either alone or in combination with inorganic phosphate. Examination of the colostrum whey or the filtrate derived from it after removal of the heat-coagulable protein showed that over 90 % of the ester phosphate could be identified by paper chromatography as glucose-6-phosphate. This phosphate ester is resistant to acid hydrolysis, a procedure which in previous experiments had not been found to abolish the accelerating effect of the whey filtrates upon globulin absorption.

In the concentrations found in colostrum whey (0·57 mM), glucose-6-phosphate had only a slight effect upon the absorption of globulin (Fig. 8 *b*) and did not alter significantly the action of inorganic phosphate when combined with it (Fig. 8 *c*). The further addition of 10 g/l. of glucose to these solutions did not affect the results.

The principal difference between the effects of the mixture of inorganic phosphate and ester phosphate and those of the filtrates of colostrum whey on globulin absorption was the large displacement of the absorptive pattern to the right; this delay resembled that found after removal of the heat-stable proteins of the filtrate by dialysis, peptic digestion or trichloro-

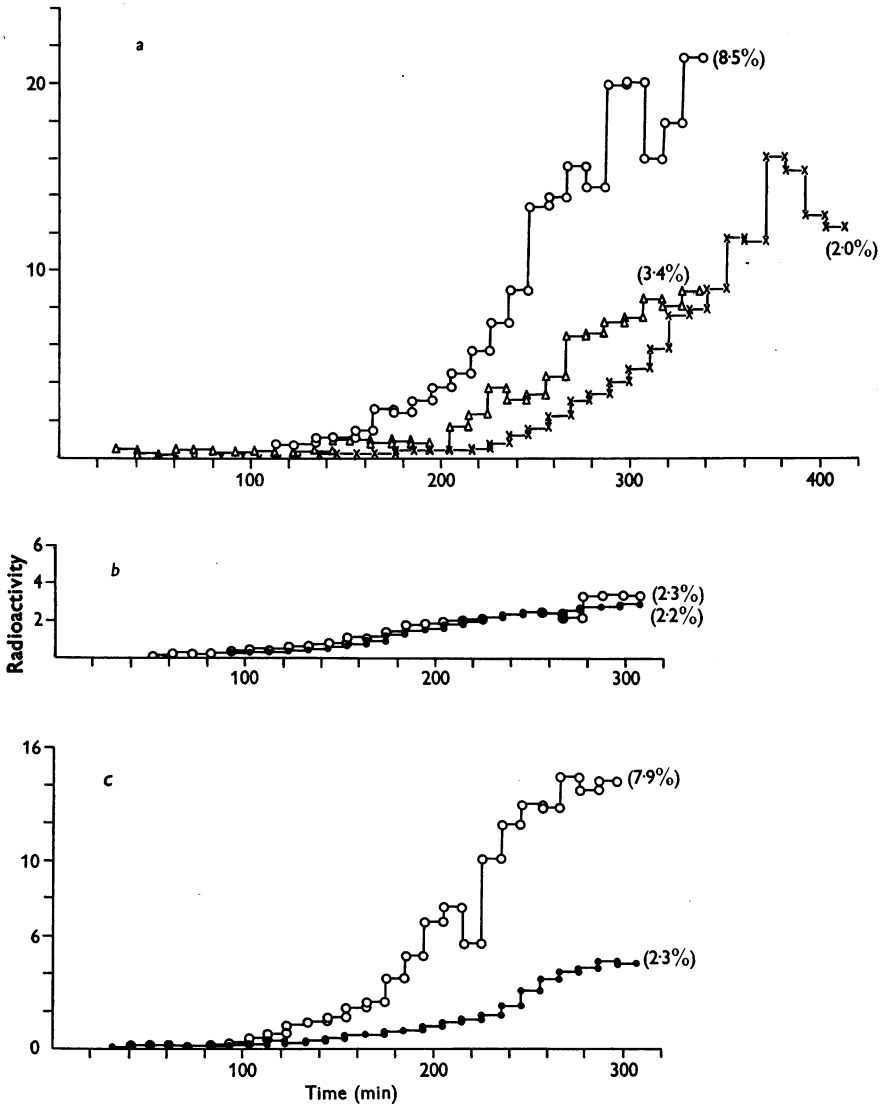

Fig. 8. Absorption of ^{131}I globulin from a 2 % solution of bovine serum globulin with the same Na, K concentrations as in whey filtrates with the following additions: (*a*) partial replacement of chloride with inorganic phosphate; (*b*) glucose-6-phosphate; (*c*) inorganic phosphate and glucose-6-phosphate. For phosphate concentrations see text. Recovery of globulin from the lymph *in 300 min* is given in brackets.

acetic acid precipitation. The effect of these heat-stable protein fractions upon the absorption of bovine serum γ-globulin was, therefore, examined in more detail.

Preparation of a protein from colostrum which accelerates globulin absorption. Two methods have been used to prepare solutions of a protein from colostrum with this property. In the early experiments the protein was prepared by saturation with ammonium sulphate of the filtrate derived from colostrum whey after removal of the heat-coagulable protein. The protein precipitate was then dissolved in water and extensively dialysed against water for 72 hr. The effect of this preparation was irregular; with some preparations a marked acceleration of absorption occurred but with others the effect was either small or absent.

A more satisfactory procedure was found to be that based on the early stages of the method of Laskowski & Laskowski (1951) for the preparation of the trypsin-inhibitor of colostrum. The method of preparation was as follows. Each litre of fresh colostrum was diluted with an equal volume of water and 1 l. of trichloroacetic acid (7·5 g/100 ml.) added to it. The mixture was heated to 80° C with constant stirring and allowed to stand at this temperature for 5 min. It was then cooled and filtered through Green's 'Hydruro' paper. The filtrate was then brought to 80 % saturation by the addition of solid ammonium sulphate and allowed to stand overnight at room temperature. The slight 'precipitate', which floated on the surface, was removed by filtration for further purification. At this stage the protein accelerated globulin absorption in some but not all of the experiments when it was added to a chloride solution. This effect was, however, greatly reduced (Fig. 9) on further purification by the following procedure.

The precipitate was dissolved in 7 volumes of water and sufficient trichloroacetic acid added to give a final concentration of 2·5 g/100 ml. This mixture was then heated to 80° C and held at this temperature for 5 min before cooling and filtration. The filtrate was then brought to 80 % saturation with ammonium sulphate and the precipitate collected by filtration.

This precipitate was next dissolved in 5 volumes of water, the pH adjusted to 6·5, and brought to 30 % saturation with ammonium sulphate. Any precipitate which appeared was filtered and discarded. The filtrate was brought to 70 % saturation with ammonium sulphate, the precipitate collected by filtration, suspended in water and extensively dialysed for 60 hr or more against water. This solution was either freeze-dried or, preferably, stored at $-20°$ C.

Solutions of protein extracted by this method only slightly increased globulin absorption when they were tested with a 2 % solution of γ-globulin in chloride (Fig. 10). The effect resembled that found with the

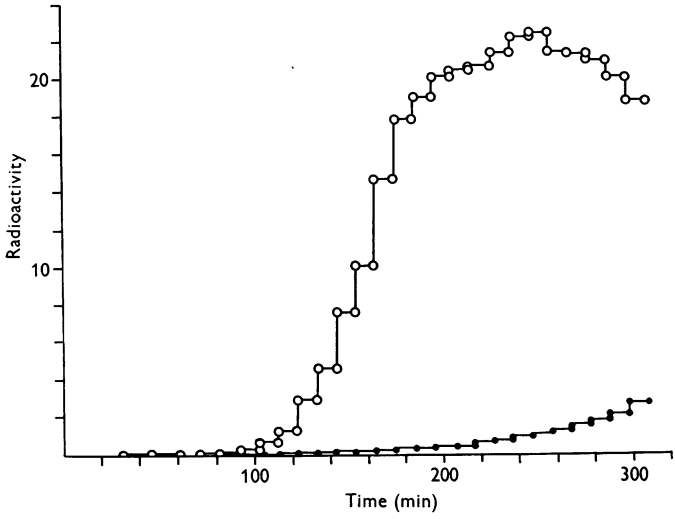

Fig. 9. The effect of the heat-stable soluble protein of whey filtrates at different stages of purification on γ-globulin absorption. A protein equivalent to 1 l. of whey filtrate was added to a 4 % (w/v) solution of serum γ-globulin with similar Na and K concentrations, as the chlorides, to that of whey. ○—○, after first stage of purification; ●—●, final stage of purification.

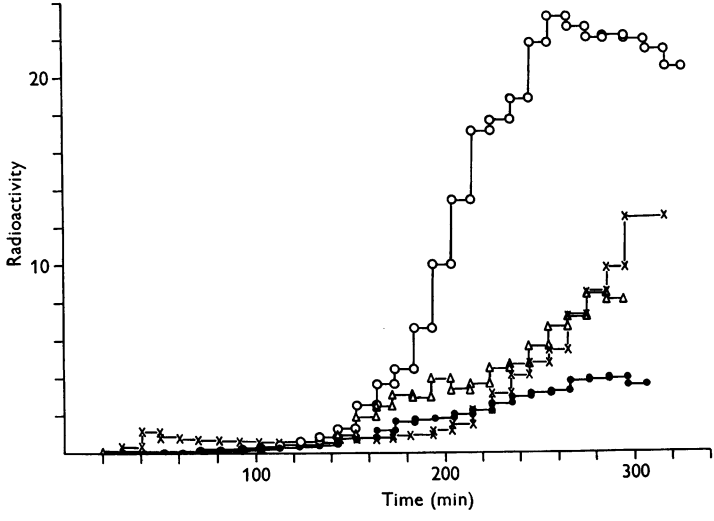

Fig. 10. The effect of the recombination of different components from filtrates of colostrum whey on the absorption of serum globulin. The same extract of a small protein fraction, stored in ampoules at −20° C, was used in all the experiments. ●—●, small protein fraction alone; ×—×, small protein fraction + glucose-6-phosphate; △—△, small protein fraction + inorganic phosphate; ○—○, small protein fraction + glucose-6-phosphate + inorganic phosphate.

non-dialysable material of the filtrates from colostrum whey (Fig. 7). This action was slightly enhanced by the further addition of either inorganic phosphate or glucose-6-phosphate (Fig. 10); the greatest effect was, however, always observed when all three components, protein, inorganic phosphate and glucose-6-phosphate, were present in the solution (Fig. 10). The addition of glucose 10 g/l. to these solutions caused a slight increase in the effect of these substances but this was neither large nor regular.

The absorption found after the addition of all three substances to the 2 % solution of γ-globulin was more rapid than that of any other recombination of factors present in colostrum whey. It was not, however, entirely equivalent to that found with fresh colostrum whey or the filtrates from it; in particular, the delay before rapid absorption became apparent was more prolonged and lasted until about 200 min and the rise in radioactivity of the lymph, when it occurred, was less abrupt.

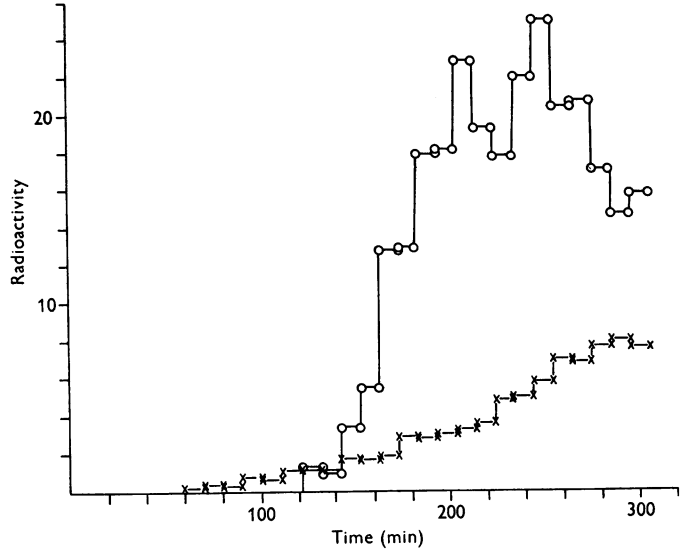

Fig. 11. Activity of filtrates from milk whey and heparinized adult bovine plasma on the absorption of serum γ-globulin. O—O, absorption from milk-whey filtrate; × — ×, absorption from adult plasma filtrate.

Filtrates of milk whey or adult blood plasma were tested for their effect on the absorption of globulin (see Fig. 12). The adult plasma was treated with heparin and the whey prepared from milk by the same procedure as colostrum. In both cases the heat-coagulable proteins were removed by filtration after boiling at pH 5·4. Filtrates derived from milk had a considerable accelerating action but the delay before this occurred was more prolonged than with colostrum. In contrast, only slight activity was found in the filtrates from adult bovine plasma.

DISCUSSION

The original observations by Howe (1921) on the appearance of globulin in the blood plasma after the feeding of new-born calves with colostrum represented the transfer of considerable amounts of unchanged protein from the lumen of the gut to the peripheral circulation. From the results of the present experiments it is clear that such a transfer, which is now known to occur across the columnar epithelial cells of the small intestine (Comline *et al.* 1951*a*; Clark, 1959), requires the presence of other substances in the colostrum and that in their absence the rate of absorption is extremely low. These additional factors are, however, only effective during the restricted period following birth in which the phenomenon normally occurs; they accelerate the absorption but are not entirely responsible for it. Changes of another type, presumably in the properties of the epithelial cells, appear to be responsible for the disappearance of this form of absorption at about 24–36 hr of age.

One of the principal points which has to be considered in relation to the results of this paper is the validity of the method used to measure the transfer of intact protein across the epithelium of the small intestine. In the present experiments the appearance of bovine serum γ-globulin labelled with ^{131}I in the lymph has been assumed to reflect directly the rate and amount of absorption of the protein. This method was based essentially on the selective entry of globulin into the lymphatic rather than the blood-vascular capillaries, on the absence of specificity in the absorption of different proteins in the young calf and lastly in the maintenance of a stable link between the ^{131}I and the bovine serum γ-globulin during its passage across the mucosa.

The present results in which a high level of radioactivity in the lymph from a cannulated thoracic or intestinal lymphatic duct was associated during absorption with extremely low levels in the blood plasma fully confirms with a more sensitive technique the previous conclusion, based on the identification of colostrum globulin by agglutination or salt precipitation, that globulin is transferred almost entirely in the lymph to the peripheral circulation (Comline *et al.* 1951*a*). Similar characteristics of the form of absorption were obtained with both types of globulin.

The absorption by the new-born calf of globulin therefore appears to differ from protein absorption in the young rat, in which a selective entry for homologous globulin has been demonstrated (Halliday, 1955; Halliday & Kekwick, 1960), and in which a large proportion of the activity of the labelled protein may be degraded to smaller molecules which can enter the blood-vascular capillaries and the extracellular fluid (Brambell, Halliday & Hemmings, 1961). There is no evidence that a similar degradation

occurs in new-born calves. Thus in the present experiments the rise in the radioactivity of the lymph can be attributed to the presence of unchanged ^{131}I-labelled serum γ-globulin for the following reasons. First, any smaller molecules produced by hydrolysis in the intestine could be expected to enter the blood-vascular as well as the lymphatic system after absorption and give rise to a substantial increase in the radioactivity of the blood. In all the experiments of this paper in which this point was tested only traces of radioactivity could be found in the blood; secondly, the radioactivity in the lymph was associated with molecules which did not pass across a cellophane membrane, which was only permeable to those of less than 10,000 molecular weight; lastly, the transfer of the ^{131}I label to other proteins during absorption is unlikely, since column electrophoresis of the lymph showed that the radioactivity was confined to the γ-globulins, the most slowly moving fraction at pH 8·6.

The initial assumptions appear, therefore, to have been justified. Examination of the lymph allowed a more quantitative assessment of the rate and extent of globulin absorption in that it avoided the dilution which occurs in the blood plasma and the effect of the variable and, as yet, unmeasured differences in the permeability of tissues to the radioactive protein. Thus the start and the course of the absorption could be determined more precisely and the quantity of protein which passed across the mucosa assessed more accurately. Complete absorption of unchanged protein by the new-born calf appears to be relatively slow even under optimal conditions; thus although the concentration of globulin in the lymph falls after the initial abrupt rise it still remains at a relatively high level for many hours. The present experiments were limited to 300 min. The amounts of protein recovered from the lymph give an indication of the possible extent of this absorption and provide a clear measure of how the rates of absorption may vary; they are not a measure of the maximal absorption, which would have required the continuation of the experiments for some hours.

The conclusion that the colostrum contains factors which enhance γ-globulin absorption is inescapable. A comparison of the absorption from the colostrum whey, either before or after the removal of the heat-coagulable protein, with that from a solution of chlorides with a similar cation composition to that of colostrum acted as the basis for subsequent work because, with the latter solvent, the absorption appeared to be minimal and the recovery in 300 min was only 1–2 % of the initial dose. Further analysis was also dependent upon the very characteristic and abrupt rise in the radioactivity which occurred in the lymph during absorption from the colostrum whey or its filtrates and which provided an excellent criterion by which to judge the actions of substances on the absorption. Two main

groups of substances, the small protein fraction and the phosphate compounds, were required for this initial rapid increase of serum γ-globulin in the lymph. Both groups remained after removal of the heat-coagulable protein from the colostrum whey. Removal of one of them, or its omission from the test solutions, altered the time course of the absorption.

The protein fraction by itself had little accelerating action on the absorption of γ-globulin; its presence was, however, essential for the full effect of the phosphate compounds. It appeared to accelerate particularly the initial stages in the absorption and in its absence the abrupt rise in radioactivity of the lymph was replaced by a more gradual increase which in a certain number of experiments reached the same final concentration but after a much greater delay. From its properties on precipitation with ammonium sulphate and other tests this protein fraction appears to have a relatively small molecular weight and to be resistant to acid and possibly alkaline hydrolysis. It is probably similar to if not identical with the relatively low-molecular-weight protein fraction which Deutsch & Smith (1957) found on electrophoretic examination was absorbed from the colostrum and then rapidly excreted in the urine. While it is now possible to ascribe a function to this protein fraction, the present experiments do not explain the mechanism by which it accelerates the globulin absorption. Three possibilities can be suggested to account for its effect. First, the much more rapid solution of the bovine serum γ-globulin in the presence of this small protein fraction was very obvious during the preparation of the test solutions. This suggests that it may act in a manner analogous to that of surface-active agents and thereby enhance the absorption. Secondly, the small protein fraction may combine with the mucopolysaccharide, which is probably contained within the vacuoles described in the intestinal epithelium (Smith, 1925; Comline *et al.* 1951*b*; Hill & Hardy, 1956), and by doing so allow it to be discharged on the lacteal side. Lastly, it may have an intracellular function not expressly connected with the absorption of γ-globulin. In the present experiments the method of Laskowski & Laskowski (1951) for the isolation of the trypsin inhibitor of colostrum has been used as a convenient method for the preparation of the comparatively large amounts of protein required for testing, but there is no evidence to suggest that the property of trypsin inhibition is essential for the acceleration of globulin absorption.

The substitution of inorganic phosphate for chloride in the test solutions enhanced the absorption. The effect, however, was slower in onset and much more irregular than that of colostrum or its filtrates. Thus, although in a certain number of experiments a similar final concentration of absorbed protein to that of colostrum was reached, the abrupt rise in radioactivity was always absent and the curve of absorption was moved to the right.

Glucose-6-phosphate, which has been identified as the most abundant component of the organic phosphate of colostrum whey, had little effect by itself or in combination with inorganic phosphate but appeared to be required for the full effect when the small protein fraction was included in the test solutions.

Inorganic phosphate has been claimed to increase the absorption of glucose in the adult (Magee & Reid, 1931), but the intestinal epithelium of the new-born calf appears to be exceptional in the size of its response to a supply of phosphate from the lumen. In contrast to this, convincing evidence could not be found that a similar supply of glucose or lactose from the lumen limited the rate of absorption, as may occur in the adult, especially in experiments with isolated intestinal sacs (Smyth & Taylor, 1958). Further evidence is required before the action of the phosphate compounds can be fully explained, but they probably act on the intracellular metabolism of the intestinal epithelium to provide the energy for absorption or more specifically for the transfer of globulin across the epithelial cells. This view is in accord with the theories of Verzar (1936). Whether other tissues of the new-born calf, in addition to the intestinal epithelium, require a similar supply of these factors in colostrum is a matter for further experimental work.

An examination of the experimental results shows that the recombination of factors identified in the whey after removal of the heat-coagulable proteins was never quite as effective as the filtrates; in particular the initial rise in radioactivity was not as abrupt. Further work is required before it can be decided whether this difference can be attributed to partial inactivation of the small protein fraction during extraction or to the need for another factor, such as a co-enzyme, which would enhance the activity of the phosphate compounds.

Colostrum, typically, has a relatively low sodium and a high potassium content. An extensive range of sodium:potassium ratios has not been examined, but it is clear that the process of globulin absorption is much more sensitive to the concentration of these cations than to that of either calcium or magnesium, which appeared to have no appreciable effect. An increased sodium concentration reduced the absorption, and the greater recovery from the caecum in these experiments suggests that it did so by preventing the entry of the globulin into the intestinal cells. This sensitivity to sodium considerably increased the difficulties in the preparation and testing of the various factors in the colostrum whey, especially that of the small protein fraction, and care had to be taken to reduce the concentration of sodium in the final solutions to levels which would not interfere with the absorption.

The sodium : potassium ratio of colostrum is closer to that of intracellular

fluid than to that of serum, and this probably applies to other components such as the phosphate compounds. This may be the explanation for the relatively poor absorption of globulin when it was dissolved in adult serum from which the heat-coagulable proteins had been removed. Whatever the reasons, the observation is important in view of the attempts to use serum as a substitute for colostrum in the new-born of certain species.

A histological examination of the small intestine was not undertaken in the present experiments, but previous observations may help to interpret the results. The absence of protein inclusion bodies in the columnar epithelial cells in the older animal in which absorption of globulin no longer takes place (Comline *et al.* 1951*b*; Clark, 1959) suggests that the barrier to it occurs at the lumen border through which the protein enters by micropinocytosis (Clark, 1959). This would support the view that the factors in colostrum which enhance absorption act primarily within the epithelial cells after passage of the protein into them. Their effect could then be expected to be limited to the restricted period following birth during which the protein may cross the brush border. Further work is, however, required to substantiate such an explanation.

SUMMARY

1. The absorption of unchanged γ-globulin from the small intestine has been examined in calves 4–17 hr of age by the use of bovine serum γ-globulin labelled with ^{131}I. The results confirmed that γ-globulin is transferred in the lymph from the small intestine to the peripheral circulation.

2. The rate and extent of the absorption were assessed by measurement of the radioactivity of the lymph. When ^{131}I serum globulin was administered into the duodenum in fresh colostrum whey, radioactivity began to appear in the lymph after 80–120 min and reached a maximum value in about 200 min. Between 12 and 25 % of the ^{131}I-labelled protein was recovered from the lymph within 300 min.

3. When ^{131}I-labelled globulin was administered in a solution of sodium, potassium, magnesium and calcium chlorides, the cations being at the same concentrations as in colostrum whey, very little was absorbed.

4. The difference between whey and the chloride solutions has been shown to be due to substances in the whey which remained after removal of the heat-coagulable proteins by boiling at pH 5·4.

5. A protein fraction, probably of low molecular weight, has been separated from colostrum whey. By itself this protein had little effect on the absorption of ^{131}I-labelled globulin, but if inorganic phosphate and glucose-6-phosphate were added, in the concentrations normally present in colostrum whey, the globulin was absorbed about as fast as from fresh

whey. In the absence of this protein inorganic phosphate had a variable and delayed effect on absorption and glucose-6-phosphate practically none.

6. Absorption from either the test solutions or from colostrum whey was delayed by the addition of sodium chloride.

7. Filtrates prepared from milk whey or adult serum after removal of heat-coagulable proteins were tested for their ability to enhance globulin absorption. Those from milk whey showed considerable activity; those from adult serum did not.

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