Polyethylene glycol as marker in piglet diets with a high dry-matter content

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1. Polyethylene glycol (PEG) of high molecular weight, used as a dietary marker, did not distribute uniformly in the water present in a high-casein diet as did glucose, a low-molecular-weight substance.

2. This distribution effect also occurred with the contents of the stomach and, to a smaller extent, with the contents of the first quarter of the small intestine of piglets given such a diet.

Since it was introduced by Hydén in 1955, high-molecular-weight polyethylene glycol (PEG) has been used extensively as a marker in studies of absorption and secretion in the alimentary tract, and is now probably the most-used water-soluble marker (Jacobson, Bondy, Broitman & Fordtran, 1963). It has the advantage of being physiologically inert, and is absorbed only to a very slight extent from the alimentary tract. It can, therefore, be fed with a water-soluble nutrient and the disappearance of the nutrient followed by measuring the change in ratio of nutrient to PEG along the tract on samples obtained by duodenal tube, by fistula or by slaughter.

In the course of using this marker for studies of sugar absorption from diets with a high dry-matter content in piglets, we observed apparent changes in the ratio of sugar to marker in the stomach contents which could not be explained by absorption or fermentation. To determine the cause of these changes in ratio, and how they affected the validity of tests using PEG as a marker, we carried out the following experiments in which solutions containing PEG together with various other substances were added to the diet, to the stomach contents and to the intestinal contents of piglets, and subsequently estimated. A preliminary note on this work has been published (Manners & Kidder, 1967).

EXPERIMENTAL

The piglets used were Large White × Wessex aged 1–3 weeks at slaughter. The rearing, slaughter and dissection were carried out as described elsewhere (Kidder, Manners, McCrea & Osborne, 1968), the small intestine being divided into four equal lengths, labelled A (duodenal end), B, C and D. These four samples and the contents of the stomach and the caecum were used. In Expt 1 the pigs were kept on the stock glucose diet throughout (Table 1). In Expt 2 the glucose was replaced by galactose for the three feeds before slaughter, the ration being otherwise the same. In the in vitro experiments to investigate the distribution of PEG and other substances in the diet, the basal diet, i.e. the diet without any sugar, was used.

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Experiments on gut contents

Effect of substances present in gut contents on PEG concentration

Expt 1. Gut contents were taken from twelve piglets and centrifuged; 2 ml of PEG solution (50 mg/100 ml) were added to 0·2 ml of supernatant liquid from centrifuged gut contents, and the mixture was deproteinized. After adding trichloroacetic acid–barium chloride reagent to a sample of the deproteinized solution the resultant turbidity was compared with that from an identical sample of PEG diluted with water (without treatment with deproteinizing reagents).

Table 1. *Composition (g/100 g) of the stock glucose diet*

Glucose	45·0	
Crude casein	28·8	Basal diet
Dried whole milk	20·0	Basal diet
Mineral and vitamin supplements*	6·0	Basal diet

*Details elsewhere (Kidder *et al.* 1968).

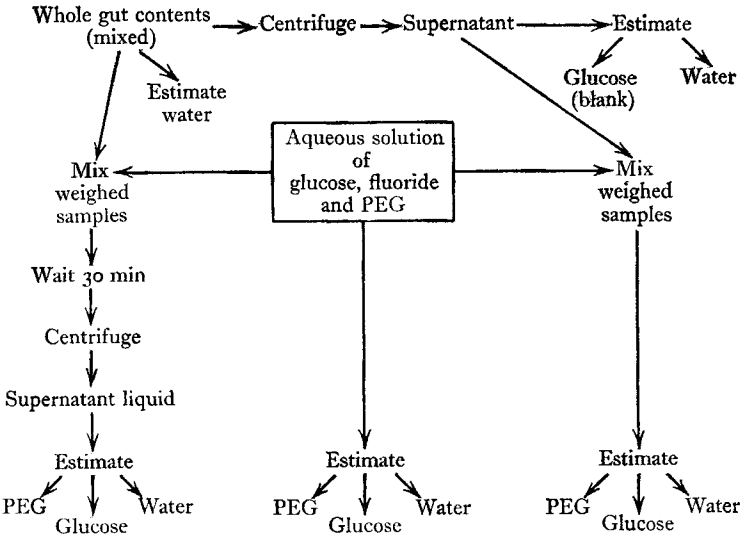

Fig. 1. Plan of the treatment of samples of stomach contents and of contents of each of the four quarters of the small intestine of piglets.

Effect of centrifuging and separation of supernatant liquid on PEG and glucose concentrations

Expt 2. Gut contents were taken from four piglets. The glucose–PEG solution used in this and in the next three experiments contained 25 % glucose, 0·42 % PEG and, to prevent glycolysis, 3·4 % sodium fluoride. Following the scheme in Fig. 1, portions (approx. 2 g) of the glucose–PEG solution were added to samples (approx. 8 g) of whole gut contents before centrifuging, and also to samples (approx. 8 g) of the supernatant fraction from centrifuged samples of the same gut contents. Glucose, PEG and water content were determined as indicated in Fig. 1. In each instance samples to be

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mixed were measured by weight, and so it was possible to calculate the theoretical concentrations of glucose and PEG to be expected in each of the mixtures.

Experiments on the diet

In Expts 3–6 the glucose–PEG solution described above was added to basal diet at the rate of 35 ml to 10 g diet, to give a mixture identical with the feed given to the piglets except for the presence of the PEG. In Expts 3 and 4, 35 ml of water were also added, diluting the mixture to a composition similar to that in the piglet's stomach 2–3 h after feeding.

Effect of temperature of mixing

Expt 3. Glucose–PEG solution was added to the diet at four different temperatures, mixed for 5 min, centrifuged at 4° and the supernatant liquid analysed.

Table 2. *Expt* 5. *The different combinations of glucose–PEG solution, basal diet and water compared*

Treatment	Blank	A	B	C	D
Basal ration (g)	3·125	3·125	3·125	3·125	3·125
Glucose–PEG solution (ml)	—	10	—	10	—
Water (ml)	10	—	10	—	10
	Stopper and mix every 2 min for 15 min				
				Centrifuge. Take 5 ml supernatant liquid	
			Add:		
Glucose–PEG solution (ml)	—	—	10	—	5
Water (ml)	10	10	—	5	—
	Centrifuge. Analyse supernatant liquid			Mix. Analyse	

Time and order of mixing

Expt 4. To one 10 g sample of diet 35 ml glucose–PEG solution were added and, 5 min later, 35 ml water. To another sample 35 ml water were added and, 5 min later, 35 ml glucose–PEG, all samples being kept at 37°. After various time intervals, samples of the mixture were taken off, centrifuged at 4° and analysed.

Expt 5. Samples of basal diet were mixed with glucose–PEG solution and water and centrifuged in different orders according to the scheme in Table 2, and glucose and PEG were estimated on the final supernatant liquids.

Comparison of distribution of PEG, inulin, glucose and urea

Expt 6. This experiment was carried out exactly as Expt 5 except that, in place of glucose–PEG solution, a solution containing 1 g/100 ml each of PEG, inulin, glucose and urea was used to compare the distribution of two high-molecular-weight and two low-molecular-weight solutes, the PEG having a molecular weight of 4000 and the inulin about 5000 (both manufacturers' specifications).

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Chromatography on a casein column

Expt 7. This experiment was carried out with the type of casein which we used in the diet, and which we regarded as the ingredient primarily responsible for the effect being studied. A 20 × 1 cm chromatographic column was prepared with a slurry of 10 g casein in 30 ml of warm water. Two millilitres of a solution containing 0·4 % PEG and 25 % glucose were put on the column and eluted with water. Successive 4 ml fractions of eluate were collected and analysed for PEG and glucose.

Analytical methods

PEG was determined by taking 0·2 ml of the material to be analysed and applying a modification of the turbidometric method described by Hydén (1955) for omasal and intestinal contents, faeces, etc. Our procedure differed from that of Hydén in two details. First, gut contents were not diluted before taking samples, but merely centrifuged, and secondly, the interval between adding the trichloroacetic acid reagent and measuring the resultant turbidity was lengthened from 5 to 50 min because after 5 min the turbidity was still increasing rapidly with time. For glucose determination the samples were deproteinized by the method of Somogyi (1930) and glucose was determined on the deproteinized fluid by the colorimetric glucose-oxidase method of Huggett & Nixon (1957). Inulin was determined by the method of Bacon & Bell (1948) and urea with a urease–phenol–hypochlorite test kit (Boehringer Corp.). In Expt 2, water content was determined by drying weighed samples to constant weight.

The 'blank' values for inulin, glucose and urea on the diet were determined as in Table 2. In Expts 3–5 the blank glucose values were less than 0·05 % of the test values and were ignored. In Expt 6, where a much more dilute glucose–PEG solution was used, the blank values for inulin, glucose and urea were about 4, 1 and 1 % of the test values respectively, and were subtracted from the corresponding test values. 'Blank' estimations for PEG were not done as the turbidity measurements are only reliable over the range corresponding to 0·05–0·1 to 0·7 mg PEG per sample (Hydén, 1955), and any turbidity due to reagents or solutes from the gut contents would not affect the comparison of whole gut contents or diet with centrifuged gut contents or diet.

RESULTS

Experiments on gut contents

Effect of substances in gut contents on PEG estimation

Expt 1. The concentrations of PEG found in gut contents are shown in Table 3 as a percentage of the values for aqueous solutions containing identical amounts of PEG. In the samples containing gut contents there was a consistent apparent elevation of the concentration of from 3 to 4 %.

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Effect of centrifuging and separation of supernatant liquid on PEG and glucose concentrations

Expt 2. If the glucose and PEG had distributed themselves throughout all the water in the samples, the determined concentrations in the mixtures would have been expected to agree with the theoretical concentrations calculated from the amounts of

Table 3. *Expt 1. The optical density of the turbidity resulting from the addition of trichloroacetic acid reagent to piglet gut contents to which known amounts of PEG had been added, as a percentage of that of the turbidity produced when the same reagent was added to aqueous solutions containing identical amounts of PEG*

(Means of results for twelve piglets)

Part of gut		Mean	Standard error of mean
Stomach		103·4	±0·2
Small intestine:*	A	103·9†	±0·5
	B	104·2	±0·5
	C	104·6	±0·4
	D	104·8	±0·4
Caecum		103·4	±0·3

* See p. 515.
† Mean for eleven samples only.

Table 4. *Expt 2. Determined levels of glucose and of PEG, and glucose : PEG ratios in mixtures with gut contents and with supernatant liquid from the same gut contents of piglets*

(Values are expressed as percentages of the calculated levels in the mixtures; means and standard errors for four experiments)

Glucose–PEG mixture added to whole gut contents and then determined on the supernatant liquid after centrifuging

		Percentage recovered		$\frac{\text{Glucose recovered}}{\text{PEG recovered}} \times \frac{100}{1}$
		Glucose	PEG	
Stomach		103·1±0·7	120·4±1·7	85·8±1·5
Small intestine:*	A	102·7±0·7	110·8±2·2	92·5±1·6
	B	102·4±0·9	108·8±2·8	94·5±2·5
	C	103·1±0·6	107·4±2·6	97·1±1·7
	D	104·2±1·0	107·3±2·6	97·5±1·8

Glucose–PEG mixture added to supernatant liquid from centrifuged gut contents and then determined

		Percentage recovered		$\frac{\text{Glucose recovered}}{\text{PEG recovered}} \times \frac{100}{1}$
		Glucose	PEG	
Stomach		100·9±0·3	108·2±2·1	94·3±1·7
Small intestine:*	A	102·2±0·6	107·9±2·1	95·8±1·4
	B	102·9±0·8	109·0±2·2	95·8±2·1
	C	104·2±0·9	108·1±2·2	97·8±1·9
	D	104·6±0·9	107·3±1·7	98·8±1·9

* See p. 515.

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glucose and PEG added and the total water content of each of the mixtures. Table 4 shows the determined concentrations of glucose and PEG in the final samples, as percentages of the calculated values. These show that when the glucose–PEG mixture was added to the supernatant liquid from centrifuged gut contents, there was little change in glucose to PEG ratio, but when the glucose–PEG mixture was added to whole gut contents which were then centrifuged, the ratio in the supernatant liquid was greatly altered in the stomach contents and somewhat altered in the contents of the first quarter of the small intestine. This alteration was due to a rise in the PEG concentration.

Experiments on the diet

Effect of temperature of mixing

Expt 3. Table 5 shows that, on addition of the glucose–PEG solution to the dry diet, followed by centrifuging, the PEG concentration of the liquid rose with negligible change in glucose concentration. As a result, a change in glucose to PEG ratio occurred, similar to that observed with stomach contents. The temperature of mixing made no difference.

Table 5. *Expt* 3. *Glucose and PEG concentrations in the supernatant liquid after centrifuging a mixture of basal diet and glucose–PEG solution mixed at various temperatures*

Material	Temperature of mixing (°C)	Glucose concentration (g/100 ml)	PEG concentration (mg/100 ml)	Glucose–PEG as % of original glucose–PEG
Original glucose–PEG	—	26·95	415·4	100
Supernatant liquid from mixture	30	25·7	458	86·6
	50	25·41	467	83·9
	70	26·05	469	85·5
	90	26·3	484	83·9

Table 6. *Expt* 4. *Composition of supernatant liquid at various times after mixing basal diet with either glucose–PEG solution and then water, or water and then glucose–PEG solution*

Time after second addition (min)	Glucose (g/100 ml)	PEG (mg/100 ml)	Glucose/PEG as % of theoretical
Theoretical concentration (original glucose–PEG × ½)			
—	13·0	205	100
Glucose–PEG solution added first			
10	12·25	233·5	82·8
20	12·23	233·5	82·6
40	12·52	235·0	84·1
Water added first			
10	12·30	229·0	84·7
20	12·29	228·5	84·8
40	12·30	228·8	84·8

Time and order of mixing

Expt 4. Table 6 gives the results of this experiment. On addition of water to the diet, followed by addition of glucose–PEG solution, or on addition of glucose–PEG

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solution to the diet, followed by water, the final PEG concentration in the liquid phase was substantially above the theoretical, while the glucose concentration was slightly below. The samples taken at different times all gave the same results, indicating that any redistribution of PEG between the solid and liquid phase was complete within 10 min.

Expt 5. The object of this experiment was to establish the cause of the alteration in PEG concentration in the supernatant phase. The concentrations found in the samples are shown as a percentage of the theoretical results in Fig. 2. An effect of the diet on

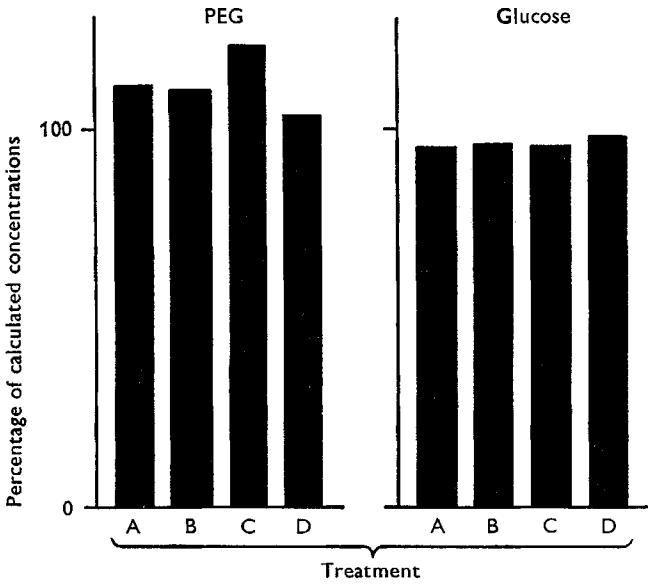

Fig. 2. Results of Expt 5. Determined concentrations of PEG and of glucose in each of the four treatments (see Table 2), expressed as percentages of the calculated concentrations.

the analytical method would have resulted in a similar change in all the samples, but would have been the only effect in sample D where the glucose–PEG was added after centrifuging. Adsorption of solute on the solid matter of the diet would have resulted in a fall in concentration in sample C which was centrifuged before dilution with water, and as a smaller fall in samples A and B which were centrifuged after addition. Greater penetration of the solid matter by the water than by the solute would have resulted in a rise in solute concentration in sample C and a smaller rise in samples A and B; precisely this effect was observed with the PEG concentrations, while no significant change was observed in glucose concentrations.

Comparison of distribution of PEG, inulin, glucose and urea

Expt 6. The PEG and glucose values in this experiment, given in Fig. 3 together with the inulin and urea values, resembled those in Expt 5. The inulin concentrations resembled those of PEG, and those of urea were similar to those of glucose. Thus, the

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two high-molecular-weight solutes rose in concentration when added to uncentrifuged diet, while the two low-molecular-weight solutes did not. To some extent, urea showed opposite changes in concentration to PEG and inulin. This may have been due to some adsorption on the solid matter of the diet.

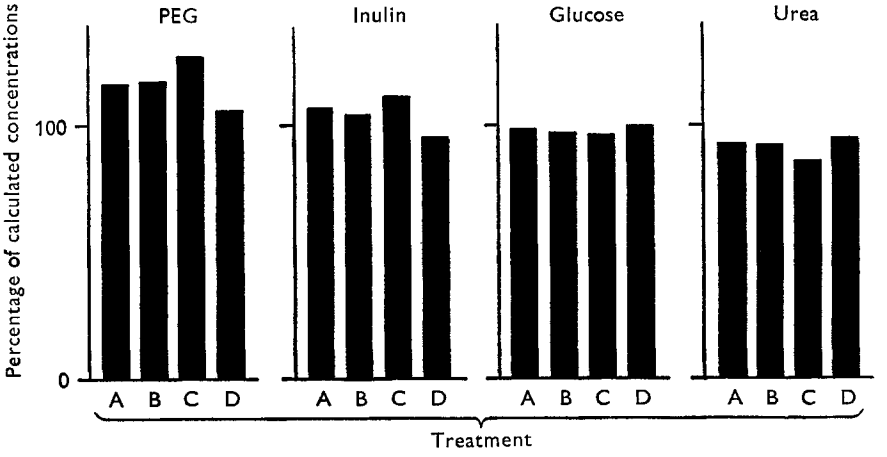

Fig. 3. Results of Expt 6. Determined concentrations of PEG, inulin, glucose and urea in each of the four treatments, expressed as percentages of calculated concentrations. See p. 517 for details.

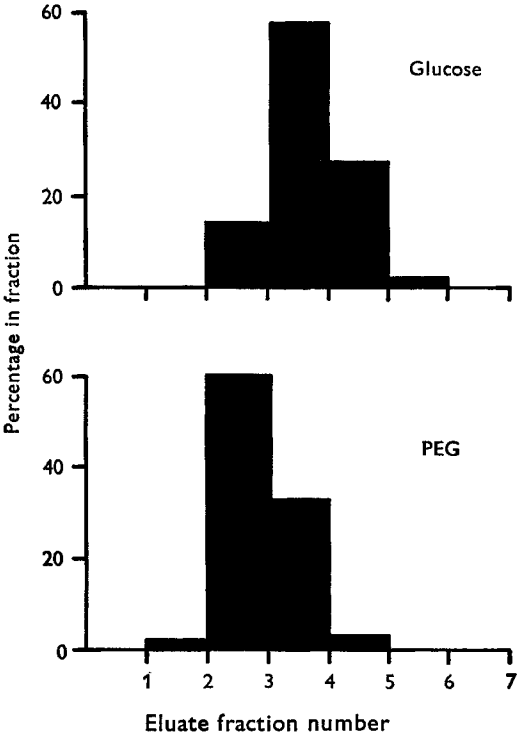

Fig. 4. Expt 7. Histograms showing the emergence of PEG and of glucose from the chromatographic column filled with casein slurry.

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Chromatography on a casein column

Expt 7. It can be seen from Fig. 4 that the PEG emerged from the column slightly ahead of the glucose, as would have been expected if a gel diffusion effect was occurring comparable with that obtained with dextran gels.

DISCUSSION

The cause of the concentration effect

Expts 3–6 showed that when a solution containing PEG and glucose was added to the piglet's basal diet, the PEG and glucose did not distribute in the same way between the solid and liquid material, so that, when the mixture was centrifuged, the PEG concentration found in the supernatant liquid was from 5 to 20% higher than the value calculated on the assumption that it distributed uniformly throughout the water present. On the other hand, the glucose concentration always corresponded closely to the value calculated on this assumption. The rise in PEG concentration could not have been a mere error in estimation due to interference from solutes from the diet, as the same interference would have occurred equally when the PEG was added to the supernatant liquid after centrifuging the diet, and under these circumstances the large rise in concentration was not observed.

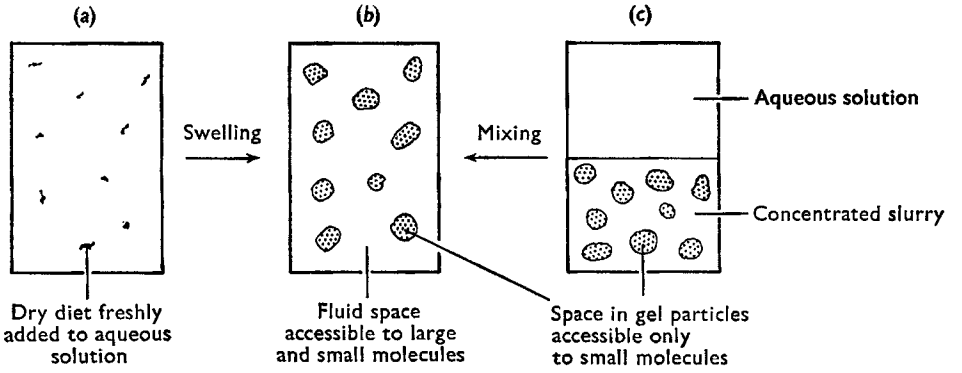

Fig. 5. Diagrammatic representation of the assumed distribution of large and small molecules within a slurry of the diet and water.

The most probable explanation for the rise in concentration of PEG in the supernatant liquid is a failure of the PEG to distribute uniformly in the water present in the wet diet, due, perhaps, to the water space in the swollen casein particles not being accessible to the high-molecular-weight PEG but being accessible to the low-molecular-weight glucose, a phenomenon known to occur in dextran gels (Porath, 1960; Andrews, 1964), in agar gels (Polson, 1961), and in polyacrylamide gels (Hjertén & Mosbach, 1962). This explanation was supported by the finding, in Expt 6, that another solute of relatively high molecular weight, inulin, behaved like PEG and another solute of low molecular weight, urea, behaved like glucose.

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The consequences of this effect are illustrated in Fig. 5. Fig. 5*b* shows diagrammatically the situation where the space in the fluid phase is accessible to both small and large molceules (in our experiment, glucose and PEG) while the space in the particles is accessible only to the small molecules (the glucose). If, as in Expt 3, dry diet is added to the glucose–PEG solution (Fig. 5*a*), the casein will take up water and swell to form particles which, on this assumption, admit glucose but not PEG (Fig. 5*b*). The result of the addition of dry diet would thus be an absolute rise in PEG concentration in the fluid phase with no change in glucose concentration. If, as in Expt 4, glucose–PEG solution is added to a slurry of the diet in water (Fig. 5*c*), then on mixing (Fig. 5*b*), the glucose concentration in the fluid phase would fall proportionately to the increase in total volume, as this would all be accessible to glucose. The PEG concentration would fall only in proportion to the increase in volume accessible to PEG. Thus the glucose to PEG ratio in the fluid phase would fall.

We can apply the same assumption (Fig. 5) to a chromatographic column made of a casein slurry. If a glucose–PEG solution were put on the column and eluted, then the PEG would start to emerge from the column as soon as a volume of water had left the column equal to the space accessible to PEG. The glucose would not emerge until a volume of water had left equal to the whole column volume. The separation of PEG and glucose on a casein column (Expt 7), with the PEG coming through ahead of the glucose, was therefore the result to have been expected if the effect was due to the molecular size of the solutes, with the swollen casein particles behaving the same way as a dextran gel towards molecules of different sizes. This experiment, therefore, confirmed our suggested explanation.

Expt 2 showed that this distribution effect of PEG occurred in stomach contents of piglets, but not in intestinal contents, with the exception of the first quarter where a small effect was found. Expt 1 made it clear that this rise in PEG concentration in the stomach contents was not due to interference in the analysis, as the large rise did not occur when PEG was added to the centrifuged supernatant liquid.

The absence of this anomalous PEG distribution in the intestine is probably due to the progressive hydrolysis of the casein so that fluid spaces between and within the casein molecules become freely penetrable to higher-molecular-weight solutes. This explanation is supported by the slight effect observed in the first quarter of the small intestine and not in the remainder, although the contents of the former part had the lowest dry-matter content.

The value of PEG as a marker

Measurements made on aqueous solutions containing PEG as a marker, infused into intestinal loops, would be little affected by the concentration effects studied here, as only low percentages of solid or semi-solid phase are present. This is one of the more frequent uses of PEG (e.g. Dahlqvist & Borgström, 1961; Schedl & Clifton, 1963). For studies in the small intestine, even in the presence of food on our type of diet, the error due to this concentration effect is within the limits of error of PEG determination except in the duodenum, and there it is only slightly greater.

In measurements made in the stomach, errors of 10–15% are likely with diets of

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the type given in the present experiments, if the contents are centrifuged and the analysis is done on the supernatant liquid. In the original method of Hydén (1955) the contents were diluted and allowed to equilibrate, and an aliquot taken and again diluted and equilibrated. This procedure will give a correct estimate of the PEG present in the sample of contents, but would not be practicable with a substance which is labile in gut contents, where speed of sampling and analysis is essential. Errors of interpretation would follow the assumption that PEG to sugar ratios in the watery fluid leaving the stomach of the animal were the same as those found in a greatly diluted, equilibrated sample used for analysis. In gastric emptying in the piglet, the fluid phase can sometimes be seen cine-radiographically to leave first (D. E. Kidder & M. J. Manners, unpublished observations), and the PEG to sugar ratio in this phase in vivo is relevant to the interpretation of PEG to sugar ratios in the lower gut. Frequency of feeding would be expected to affect the size of the errors, very frequent small feeds being likely to lead to an approximation to a steady state in output from the stomach of marker and marked substance; infrequent large feeds to the reverse.

Table 7. *Properties of an ideal marker for water-soluble substances*

Should	be well-defined chemically and homogeneous
	be soluble
	have similar 'diffusion space' to test substance
	be measurable with accuracy at low concentrations
Should not	pass through gut wall
	be taken up, adsorbed or degraded by gut flora, contents or mucosa
	influence the activity of gut flora, contents or mucosa
	influence intestinal motility

Sperber, Hydén & Ekman (1953), Smyth (1961), Fordtran (1966) and Schedl (1966) give different lists of requirements for satisfactory marker substances for intestinal studies on water-soluble substances. Only Schedl mentions the necessity that the marker should have the same diffusion 'space' as the marked substance, but his list is otherwise incomplete. From the requirements given in these four papers a more comprehensive list can be made (Table 7) of the requirements for a satisfactory marker substance for water-soluble materials. It is optimistic to expect any marker to fulfil all these requirements, but it should usually be possible to select a marker adequate for any one specific investigation.

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