# The preparation of tritium-labelled polyethylene glycol and its use as a soluble rumen marker

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In the study of ruminant digestion it is frequently necessary to determine the fluid volumes and rates of passage of fluid through the gastro-intestinal tract. Polyethylene glycol (PEG) of molecular weight 3000–3700 was introduced as a soluble reference material (marker) by Sperber, Hydén & Ekman (1953). Since then PEG has had extensive use as a soluble marker, but has at times proved difficult to estimate. Downes & McDonald (1964) stated that the lack of a specific, sensitive, and accurate method of analysis for PEG was a serious limitation in its use. These authors used the chromium-51 complex of ethylenediamine tetraacetic acid ($^{51}$CrEDTA) as an alternative, and probably equally good, soluble marker. However, in many experiments, there is need for two separate soluble markers. Though a large amount of work has been done with PEG, it is probable that, if an accurate and specific analytical method were available, this material would have even more use as a soluble marker. The labelling of PEG with $^{3}$H or $^{14}$C would overcome the analytical problem; the sensitivity of radiochemical techniques would permit the marker to be determined over a much greater range of concentrations, and consequently would extend the time during which useful information could be obtained.

This paper describes the preparation of [$^{3}$H]PEG and its use as a soluble marker. The results obtained by radioassay are compared to those obtained by a chemical method on portions of the same samples.

## EXPERIMENTAL

### *Labelled materials*

$^{3}$H gas and n-[1,2-$^{3}$H]hexadecane were obtained from the Radiochemical Centre, Amersham, UK.

Unlabelled PEG 4000 (Carbowax; Carbide & Carbon Chemicals Corporation, USA, mol. wt 3200–3700) was ground, sieved (80–100 mesh), and dried under highly reduced pressure. Some of the dry PEG 4000 (1·95 g) was exposed for 28 days to $^{3}$H gas (1·2 c, 0·48 ml at s.t.p.) in a glass ampoule fitted with a break-seal (Wilzbach, 1957). The exchangeable $^{3}$H was removed by dissolving the crude labelled material in water, which was then distilled off. This process was repeated until no more $^{3}$H was detectable in the distillate. A solution of [$^{3}$H]PEG 4000 (*c.* 1·5 g, 4·0 mc in 60 ml) was recovered. A portion (10 ml) was purified by gel-filtration (Porath & Flodin, 1959) on



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a column (100 × 1·6 cm) of Sephadex G-25 (Pharmacia, Uppsala, Sweden) with water as the eluant. The eluate was collected in 1·5 ml fractions, samples (0·01 ml) of which were assayed for $^3H$ by liquid scintillation counting (see p. 437). The eluates in tubes 19–31 (Fig. 1) were pooled and portions used for the rumen marker experiments. The other peaks contained material of lower molecular weight produced by radiation degradation of the PEG 4000.

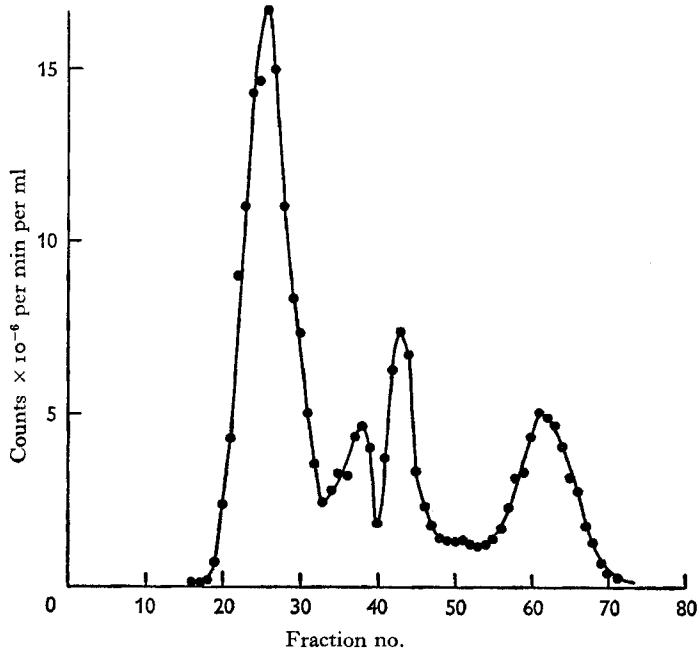


Fig. 1. Distribution of $^3H$-labelled material when crude [$^3H$]PEG 4000 was eluted from a Sephadex G-25 column.

### *Dosing*

Aqueous solutions containing [$^3H$]PEG 4000 (0·05–0·15 g; 1·5 mc/g) and unlabelled PEG 4000 (*c.* 10 g) were mixed and quantitatively injected into the rumen of a sheep.

### *Sheep*

A Merino–Border Leicester cross-bred wether, fitted with a rumen fistula, was used. It was housed indoors in a metabolism cage of the type described by Till & Downes (1963), at a temperature of 21 ± 2°. Unless otherwise stated, the sheep was given once daily (09.00 h) a mixture of equal parts of wheaten and lucerne chaff (700 g) and allowed water *ad lib.*

### *Sampling*

A device for sampling rumen liquor was made from a ½ in. outside diam. polyethylene tube, a glass trap, and a rubber suction bulb. The end of the tube which was inserted into the rumen was sealed off and the lower 1½ in. perforated with ⅛ in. diam. holes which acted as a filter for coarse particulate matter which would otherwise have

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clogged the trap. During the sampling the tube was moved around inside the rumen to get as representative a sample as possible. The sample was filtered through a plug of cotton-wool and centrifuged (10 min at 11 500 *g*). The supernatant liquid was analysed for PEG and for $^3$H.

Daily collections of urine and faeces were made. The total amounts were recorded and representative samples taken. The urine was centrifuged (10 min at 11 500 *g*) and the supernatant liquid analysed for $^3$H. The faeces samples (*c.* 50 g) were broken up in a macerator. One portion was used for the determination of dry matter, and other portions were macerated with known weights of water, centrifuged and the supernatant liquid was analysed for PEG and for $^3$H.

## *Chemical analysis* — *Determination of PEG*

It has been shown that the recovery of PEG from faecal samples required at least a tenfold dilution with water before the precipitation of protein and sulphate (Corbett, Greenhalgh, Gwynn & Walker, 1958). Consequently, in these experiments all samples were diluted with water, thoroughly mixed and centrifuged. The supernatant liquid was analysed for PEG by the method of Hydén (1956*a*) as modified by Downes & McDonald (1964).

### *Determination of $^3$H*

*General.* $^3$H was determined using a Packard Tri-Carb liquid scintillation spectrometer (Model 314EX), under balance-point conditions (Packard operation manual; Packard Instrument Company Incorporated, Box 428, La Grange, Ill., USA). The quenching of scintillation within the sample was monitored by calculation of the ratio of the counts recorded in the two channels. When variable quenching was indicated by a change in this ratio, the samples were 'spiked' with a known quantity of n-[1,2-$^3$H]hexadecane of known specific activity, and recounted. The efficiency of counting (2–5%) was determined, and the original counts were then corrected for variations in counting efficiency. The quenching was found to be greatest in samples of rumen liquor taken soon after the sheep had eaten its daily ration and progressively decreased in samples taken during the rest of the 24 h period. The quenching was probably not quite constant even in samples taken after a 24 h fast. Some of the quenching was probably due to pigmented material that was insoluble in the scintillator solution and slowly settled out, thus reducing the quenching. Consequently it is better to allow this material to settle out (*c.* 24 h) before counting the sample.

*Direct counting.* Samples (0·01 ml) of the column eluate collected during the purification of [$^3$H]PEG, and of the pooled peak fractions, were added to 2·0 ml of ethanol and 2·0 ml of scintillator solution (2,5-diphenyloxazole, 0·4% (w/v) + 1,4-bis-2(5-phenyloxazolyl)-benzene, 0·01% (w/v), in toluene). Duplicate or quadruplicate counting samples were also made up using 1·0 ml portions of rumen liquor, urine, or faeces extract, 8·0 ml of ethanol and 9·0 ml of scintillator solution.

When variable quenching of samples was suspected two of the quadruplicate samples were 'spiked' with n-[1,2-$^3$H]hexadecane and counted with the rest of the samples.

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*Preparation and counting of oxidized samples.* To check the direct counting method, a series of samples was prepared and oxidized by the oxygen-flask technique essentially as described by Kalberer & Rutschmann (1961). Samples (1·0 ml) of urine and rumen liquor were put into small sacks made from dialysis tubing, and evaporated to dryness under an infrared lamp. The dry sample was then wrapped in a piece of tissue paper and burnt in a flask which contained ethanol (5·0 ml at −80°). After the water produced by the combustion had been absorbed by the alcohol, a sample (4·0 ml) was withdrawn and placed in a counting vial together with 9·0 ml of the previously mentioned scintillator solution, and the radioactivity measured. Samples (100 mg) of dried faeces were also analysed by the oxygen-flask method.

### *Rumen marker experiments*

Two experiments were conducted in which the rate of disappearance of [$^3$H]PEG 4000 from the rumen and its recovery from the excreta were determined by both chemical and radioactivity measurements.

In the first experiment, the sheep was deprived of food and water for 24 h before the administration of [$^3$H]PEG (9·15 g, 193 $\mu$c). The sheep was allowed food and water 14 h after the beginning of the experiment and was then fed according to its normal schedule. Additional rumen liquor samples were taken at 48 and 72 h after injection of the marker into the rumen but owing to the dilution of the PEG these samples were analysed only for radioactivity.

In the second experiment, the sheep was fed at the normal time, and 2 h later the [$^3$H]PEG (10·3 g, 61·5 $\mu$c) was injected.

The rumen liquor volumes were calculated by extrapolation, to zero time, of the semi-logarithmic plots of concentration and specific activity against time. The disappearance half-times were calculated from the slopes of these curves.

## RESULTS

The results of the rumen marker experiments show that there was no significant difference in the disappearance rate, rumen volume estimate, or total recovery of marker, as determined by the chemical or radioactivity measurements (Fig. 2, Tables 1 and 2). The ratio of the concentrations of tritium and of PEG in the rumen liquor remained constant within experimental error (Table 1).

After the frequent rumen sampling in Expt 1 was finished and the sheep had returned to a normal feeding routine, the mean half-time for the disappearance of $^3$H from the rumen liquor became about 10 h (Fig. 3).

## DISCUSSION

The results show that PEG can be labelled with $^3$H by the Wilzbach (1957) technique and purified easily to give a labelled substance which, except for its radioactivity, is indistinguishable from the starting material in its behaviour as a rumen marker. The constancy of the ratio of $^3$H to PEG during the rumen marker experiments shows that $^3$H was not lost by exchange reactions under these conditions.

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PEG 4000 has previously been labelled with $^3$H by Ghanem & Westermark (1962) by a modification of the Wilzbach technique, but was apparently only used as a test substance for the labelling technique and was not purified. The present results show, however, that the purified [$^3$H]PEG prepared by the original Wilzbach technique has a sufficiently high specific activity for use as a rumen marker.

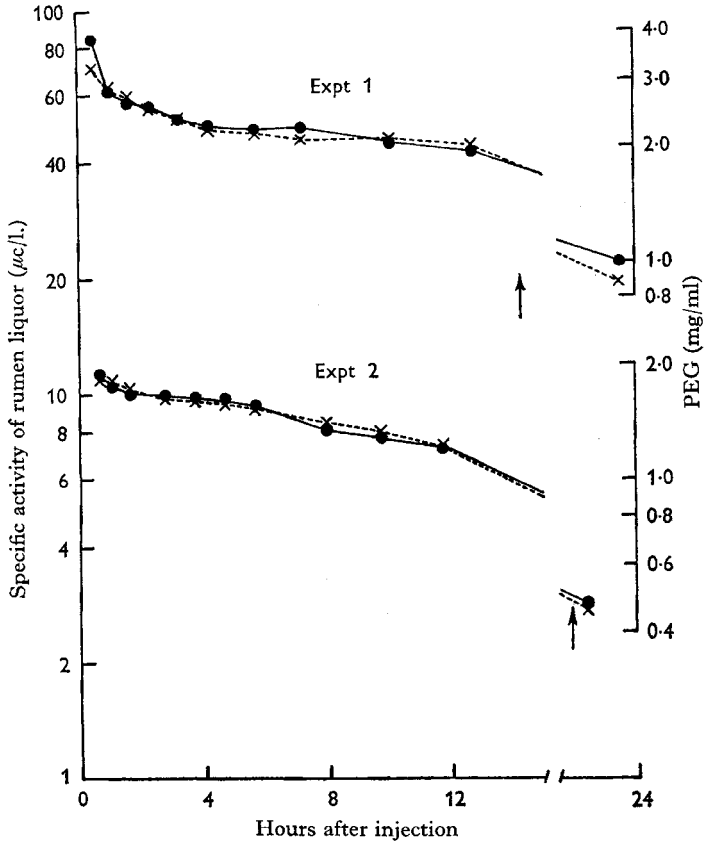


Fig. 2. Disappearance of PEG 4000 and of $^3$H from the rumen of a sheep. × - - - - ×, concentration of PEG 4000 in rumen liquor determined chemically; ●———●, specific activity of rumen liquor determined by liquid scintillation counting. The arrows show time of feeding.

Table 1. *Comparison of results based on chemical analysis and on radioassay when [$^3$H]PEG 4000 was used as a rumen marker in a sheep*

| Expt no. | Specific activity of [$^3$H]PEG injected into the rumen (μc/g) | Ratio, $^3$H:PEG in rumen liquor samples* (μc/g) | Volume (l.) of rumen liquor calculated from: Chemical analysis | Volume (l.) of rumen liquor calculated from: Radioassay | Rumen liquor half-time (h) calculated from: Chemical analysis | Rumen liquor half-time (h) calculated from: Radioassay |
|---|---|---|---|---|---|---|
| 1 | 21·1 | 22·1 ±0·7 | 3·7 | 3·6 | 37 | 36 |
| 2 | 5·99 | 5·97±0·15 | 5·7 | 5·6 | 19·5 | 19 |

* Value with standard deviation.

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The labelling with $^3H$ largely solves the analytical problem previously associated with the use of PEG. The methods of assay for $^3H$ are specific, accurate and easy. Relatively small amounts of [$^3H$]PEG may now be used in rumen marker experiments

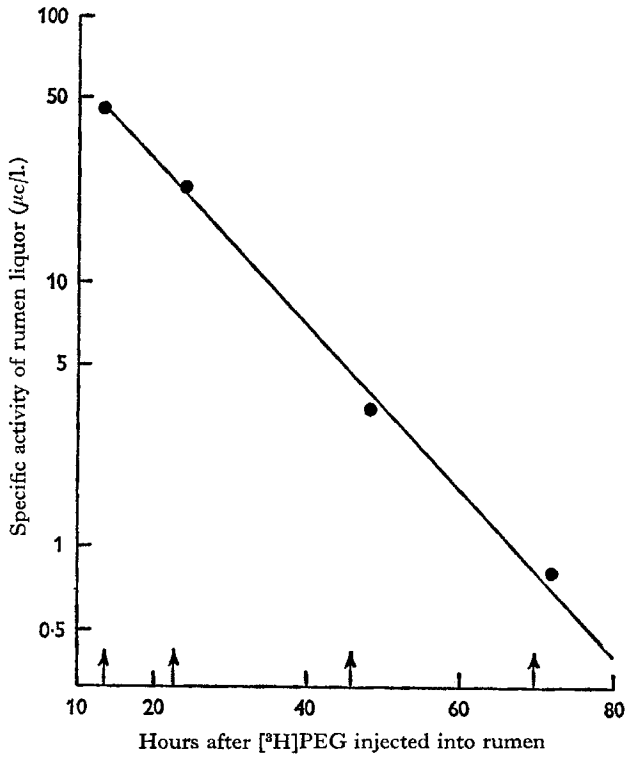


Fig. 3. Expt 1. Disappearance of [$^3H$]PEG from the rumen of a sheep during the interval 12–72 h after intraruminal injection of [$^3H$]PEG 4000. The straight line was fitted by eye and the arrows show time of feeding.

Table 2. *Percentage recoveries of $^3H$ and PEG (chemical analysis) after intraruminal administration of [$^3H$]PEG 4000 to a sheep*

| | | Expt 1 | | Expt 2 | |
|---|---|---|---|---|---|
| Sample | Day | $^3H$ | PEG | $^3H$ | PEG |
| Faeces | 1 | 5·4 | 8·6 | 0·0 | 0·0 |
| | 2 | 27·3 | 28·3 | 70·3 | 68·0 |
| | 3 | 37·9 | 38·6 | 13·1 | 10·5 |
| | 4 | 7·6 | 9·1 | 6·6 | 5·3 |
| | 5 | 0·0 | 0·0 | 0·0 | 0·0 |
| Urine | 1 | 1·4 | — | 1·3 | — |
| | 2 | 1·1 | — | 0·5 | — |
| | 3 | 0·5 | — | 0·1 | — |
| | 4 | 0·2 | — | 0·0 | — |
| | 5 | 0·0 | — | — | — |
| **Rumen liquor** | | 13·5 | 12·6 | 4·2 | 4·8 |
| Total | | 94·9 | 97·2 | 96·1 | 88·6 |

—, not measured.

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and can be detected over much longer periods of time compared with unlabelled PEG. In these experiments the unlabelled PEG 4000 was added to enable chemical determination of PEG to be carried out. However, even when chemical measurements are not required, the use of PEG of low specific activity would probably be desirable to reduce any errors due to possible absorption of a small quantity of PEG by the rumen contents.

The recovery of PEG from the faeces was in the expected range for ruminants as reported by Hydén (1956*b*), Corbett *et al.* (1958) and Downes & McDonald (1964). The appearance of $^3H$ in the urine is not surprising, as small quantities of PEG were detected by Downes & McDonald (1964) but could not be determined quantitatively owing to the limited sensitivity of the analytical technique used. The $^3H$ detected in the urine may not be present as PEG but it is certainly still in a non-volatile form. In experiments with cows, Hydén (1956*b*) observed lower recoveries of PEG when the marker was administered intraruminally than when it was administered into the abomasum, even though PEG is not destroyed by rumen micro-organisms, or by standing for days in urine or faeces (Sperber *et al.* 1953; Hydén, 1956*b*; Corbett *et al.* 1958). The form in which PEG passes out of the rumen is not known, although when it is injected intravenously into man 96% is excreted in the urine in 12 h (Shaffer & Critchfield, 1947).

The half-time (about 10 h) for the disappearance of $^3H$ from the rumen, observed over the 3-day period in Expt 1, probably represents the mean half-time of the rumen liquor under the normal feeding routine. The long half-time (37 h) and lower rumen liquor volume (3·6 l.) calculated for the first 14 h of this experiment were probably the result of starving the animal. The cause of the long half-time (19 h) in the second experiment is unknown, but it may have been partially due to the sampling.

PEG 20M (Carbowax, mean mol wt 20000) has also been labelled by the Wilzbach gas exposure technique; the exchangeable $^3H$ and by-products of low molecular weight were removed by dialysing against demineralized water and the [$^3H$]PEG 20M was purified on a column of Sephadex G-50. When samples of the [$^3H$]PEG 20M (*c.* 40 $\mu$c in 2 g) and $^{51}$CrEDTA were used in rumen marker experiments with sheep, both substances gave essentially the same results (A. C. I. Warner, personal communication).

By the use of the appropriate radioassay technique it should be possible to assay samples for mixtures of [$^3H$]PEG and $^{51}$CrEDTA, although the weak X-rays from $^{51}Cr$ complicate the analysis of the mixture for $^3H$. Thus the use of both of these markers should make easier the measurement of the rumen liquor volume and half-time when repeated measurements are required at intervals that are short compared with the half-time.

## SUMMARY

1. Polyethylene glycol (PEG 4000) was labelled with tritium by direct exposure to $^3H_2$ gas and purified by gel filtration.

2. The [$^3H$]PEG was used as a soluble marker in two experiments on gastro-intestinal function with one Merino–Border Leicester cross-bred wether provided with a rumen fistula. The disappearance of the marker from the rumen and its

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recovery in the excreta were measured both by liquid scintillation counting and by a chemical method.

3. The two methods gave the same results within experimental error for the rumen volume estimate, rate of disappearance from the rumen, and for the recovery in the faeces.

4. A small amount of $^3H$ (2–3% of the dose) was recovered in the urine but the compound in which it was present was not chemically identified.

5. It is concluded that PEG can be readily labelled with $^3H$ to give a substance which, except for its radioactivity, is indistinguishable from unlabelled PEG in its behaviour as a rumen marker.

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