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The Fate of Tritiated rm-Epidermal Growth Factor in the Sheep: Validation of the Labelling Procedure and Rate of Tissue Clearance

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Abstract

Plasmid-derived recombinant mouse epidermal growth factor, rm-EGF, was purified by ion pair reversed phase high performance liquid chromatography. The product peak (termed rm-α-EGF) was characterized by physicochemical techniques including fast atom bombardment mass spectrometry, high field proton magnetic resonance and amino acid sequencing (amino acid arrangement and composition). The rm-α-EGF was tritiated, labile tritium removed by lyophilization, and the product purified and characterized as for the parent compound to yield a compound identical to rm-α-EGF except for the isotopic hydrogen substitution. Label stability was validated by lyophilization of samples, especially urine.

The tritiated rm-α-EGF was used to determine the excretion rate and tissue distribution pattern in the sheep. It was administered by intravenous infusion for 24 h at a dose rate of 120 μg kg^{-1} live weight. Blood, urine and faeces were collected at frequent intervals from all sheep up to slaughter. Sheep were slaughtered at 24 h (3 sheep), 48 h (3 sheep) and 192 h (1 sheep) from the start of infusion and samples of all tissues and organs collected. Samples were assayed by liquid scintillation counting, directly for liquids, and after combustion to tritiated water for solids. For residue studies all solid samples were lyophilized to constant weight before combustion, and volatile tritium determined from the lyophilisate. Urinary excretion was extensive and rapid. From the start of the infusion 30·1% of the administered tritium was recovered at 24 h, 40·4% at 48 h and 55·1% at 192 h. Comparison of RIA and tritium (^{3}H) in plasma and urine samples indicated that the EGF had undergone considerable metabolism. Faecal excretion of EGF was also significant, being 1·5% at 24 h, 2·1% at 48 h and 10·0% at 192 h after the start of the infusion.

Of the EGF not excreted at the time of slaughter, 41·9% (24 h), 36·8% (48 h) and 22·1% (192 h) was present in eight locations: muscle, intestine, gut content, skin, blood, liver, kidney, and lung. Tritium in fat (omental, perinephric, subcutaneous) was negligible, and no ^{3}H was detected in the plucked fleece 192 h after the start of the infusion. Volatile metabolic products (H_2O, CH_4, NH_3) excreted via the lung were not measured.

The overall recoveries of 97·4% (24 h), 100·5% (48 h), and 97·8% (192 h) confirm that the label was in stable positions. This result thus validates the labelling procedure and the use of a *generally* labelled compound, and confirms the efficacy of the sampling procedure. Complete inhibition of wool growth, as shown by wool shedding 192 h after the start of the infusion indicated that this biological activity of the compound was not affected by the labelling procedure.

These residue data were used to assess the risk to humans from the consumption of meats and offal from sheep treated with EGF. It was concluded that no harmful effects were likely.

Introduction

Growth factors have been isolated from the mouse (Cohen 1960; Levi-Montalcini and Cohen 1960), humans (Gregory 1975), the rat (Simpson *et al.* 1985), the shrew (Yip *et al.* 1985) and deer antlers (Ko *et al.* 1986). Murine epidermal growth factor (mEGF) isolated

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from the submaxillary glands of the mouse was amongst the first of such factors to be isolated and it has been characterized and its biological activity extensively investigated (Cohen 1962). It is a 53 amino acid residue polypeptide with both amino acid sequence and composition reported (Savage *et al.* 1972). It has a molecular weight of 6040 (O'Keefe *et al.* 1984; Hyver *et al.* 1985).

EGF is currently under investigation as a wool harvesting agent in the sheep (Panaretto *et al.* 1982). An important aspect of this investigation was to determine the excretion pattern of EGF and the location of EGF residues (including metabolites) in various organs of dosed sheep. This information is essential when a compound is proposed for administration to animals which form an important part of the human diet especially since EGF is known to be a powerful mitogen (King and Carpenter 1983). Such information can also be used in developing a higher grade hypothesis on the mode of action of the compound and for structure-activity correlations. Because of the small amounts of compound involved (μg kg^{-1} body weight) a radio-labelled compound was the only feasible method of carrying out such a study.

This project required a source of pure, characterized EGF; a method of labelling this EGF with a radionuclide; and a technique for purifying the product of the labelling reaction, in which the biological activity under investigation i.e. wool-growth inhibition, was retained.

The EGF used for the project was plasmid-derived mouse EGF (rm-EGF) produced by recombinant DNA technology (Allen *et al.* 1987). It was separated by high performance liquid chromatography (HPLC) into three fractions of which the major component, rm-α-EGF (80%), was shown by structure determining techniques to be identical to m-α-EGF which is the major component of EGF recovered from the submaxillary glands of the mouse (O'Keefe and Sharry 1986). The tritium (^{3}H) labelling was carried out on this rm-α-EGF.

For isotopic labelling of EGF without synthesis, only ^{3}H is feasible. We have developed a modified method for the radiation induced tritiation of the intact EGF molecule in the presence of a catalyst (Garnett and O'Keefe 1975*a*, 1975*b*). The labile ^{3}H was removed from this product by lyophilization, and the product purified by ion pair reverse phase high performance liquid chromatography (IP RP HPLC). The compound was then characterized as before (O'Keefe *et al.* 1984). Previous batches of the tritiated compound had also been evaluated in several *in vivo* studies in the sheep and found to be a valid tracer for EGF (J. H. O'Keefe, L. F. Sharry and B. A. Panaretto, unpublished data). The ^{3}H-rm-α-EGF was used to determine the excretion pattern and undifferentiated tissue residues in sheep infused intravenously.

Non-isotopic labelling with ^{125}I, which would have had the advantage of simplifying the sample counting procedure, was considered and rejected because the large foreign iodine atom is not necessarily a tracer for the EGF molecule. Additionally, the iodination procedure has been shown to produce a mixture with EGF and similar peptides (Magun *et al.* 1982). From such a mixture it would be difficult to separate and characterize a compound guaranteed to be ^{125}I-EGF. Such an uncharacterized compound, however, has been used in location studies in the rat (Thornburg *et al.* 1984).

Materials and Methods

Experimental Animals

Eight mature fine wool Merino wethers each weighing approx. 35 kg were kept in metabolism cages in a room controlled between 20 and 24°C. The ration was 600 g of pelleted food (lucerne 60%, oats 40%) given once daily (0900 h). Drinking water was available *ad libitum*. Faeces and urine were quantitatively collected and subsampled.

Experimental Procedures

Purification of EGF

The plasmid-derived rm-EGF was obtained from Coopers-Wellcome (Aust.) and purified by IP RP HPLC. The separation was carried out isocratically using a semi-preparative C18 μ Bondapak column

(30 cm × 7·8 mm) (Waters Assoc.) and a 6000A solvent delivery system with a model 440 fixed wavelength dual channel u.v. absorption detector (Waters Assoc.). The mobile phase was acetonitrile : water (26 : 74), 0·04 M in triethylamine acetate, pH 5·6. All samples and solutions were passed through an 0·5 μm filter (Millipore) before loading on to the model U6K universal injector. Detection was by u.v. at 254 nm and with selected samples, by differential refractometry.

The peak components were recovered by lyophilization and the major peak termed rm-α-EGF.

Preparation of tritiated EGF

100 mg of the purified rm-α-EGF was tritiated in the presence of a palladium catalyst by a modification of the method of Garnett and O'Keefe (1975*a*). The reaction product was dissolved in water, adjusted to pH 8 with sodium hydroxide and filtered. Labile ^{3}H was removed by repeated lyophilization and the product purified by IP RP HPLC as before. This product was again checked for labile ^{3}H by lyophilization. This material had the same retention time as rm-α-EGF and was characterized by instrumental methods (see below). Its specific activity was determined by liquid scintillation counting.

Characterization of EGF

The instrumental characterization of the rm-α-EGF and its tritiated form (^{3}H-rm-α-EGF) were essentially as described by O'Keefe *et al.* (1984). Molecular weight was determined by fast atom bombardment mass spectrometry (FABMS) (VG analytical ZAB-HF). Mass assignments for the compounds were made on peak centroids. High field proton magnetic resonance (HFPMR) was carried out using a HFX-270 unit (Bruker) in the Fourier transform mode. Ultraviolet spectrophotometry was carried out with a DMS 100 spectrophotometer (Varian) over the wavelengths range 250–300 nm. Optical activity was determined on a model 241 polarimeter (Perkin Elmer) equipped with both sodium (589 nm) and mercury (578 nm) lamps.

Automated Edman degradations were performed for amino acid sequencing using a model 470A vapour phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.) according to the standard protocol. The sequence was terminated after 55 cycles, and the amino acids sequenced were identified using reverse phase HPLC and the phenylhydantoin (PTH) amino acid derivatives.

Amino acid analysis was carried out in conjunction with the sequencing. Identification and quantitation of the amino acids were by HPLC using the Waters PICO-Tag method and acid hydrolysis times of 24 and 72 h (Hewick *et al.* 1981; Bidlingmeyer *et al.* 1984). Radioreceptor assays were performed as one determinant of retention of biological activity. The receptor binding affinity of rm-α-EGF, ^{3}H-rm-α-EGF and mEGF were compared using radioiodinated EGF as ligand (Wynn *et al.* 1986).

Preparation and infusion of dose

Doses were prepared from a stock material obtained by mixing 37 mg of ^{3}H-rm-α-EGF and 15 mg of untritiated rm-α-EGF, dissolving in water, filtering (0·5 μm), and lyophilizing to obtain a homogeneous product. This product was stored in a vacuum desiccator over silica gel at 2°C. The dose rate was 120 μg kg^{-1} of fleece-free body weight and this was administered in 0·9% pyrogen-free sodium chloride. Individual weighed doses were prepared for each sheep depending on its estimated fleece-free weight and on the delivery rate of the infusion pump used. An aliquot of each dose was lyophilized to monitor labile tritium.

For the infusion, two catheters (i.d. 1 mm, o.d. 1·5 mm) were inserted into each jugular vein several days before infusion. One, passed caudally, was used for infusing the ^{3}H-rm-α-EGF and the other, passed cranially, was used to collect blood samples.

Infusions were carried out using a syringe pump (Harvard) delivering approx. 1 ml h^{-1}.

Total amounts administered varied from 3·9–5·4 mg. Doses on average were 4·2 mg EGF which contained 1·554 MBq (42 μCi) ^{3}H-rm-α-EGF in 30 ml 0·9% sodium chloride.

All infusions were carried out for 24 h, the residual content of the syringes weighed and the administered dose calculated.

Animals were slaughtered at the following times from the start of infusion: 24 h (3 sheep), 48 h (3 sheep) and 192 h (2 sheep). There was a problem with the infusion of one of the 192 h sheep and the duplicate for that period was rejected.

Sampling and Processing

In vivo *samples*

Blood, urine and faeces were sampled and stored at −20°C until assay. All assays for ^{3}H were by liquid scintillation counting.

(i) *Blood*

10 ml of blood was collected using an heparinized syringe and centrifuged at 2°C and 6000 *g* for 10 min. Collection times were as shown in Fig. 2. The plasma layer was transferred to a container and stored at −20°C. The red blood cells were also retained and assayed after combustion in a 306 Oxidiser (Packard) (see below).

(ii) *Urine*

The total urine voided was collected and the collection time and volume recorded (Fig. 3). For each period a 100 ml subsample was stored at −20°C without stabiliser.

(iii) *Faeces*

The complete output was collected by an attached plastic bag, removed once per day, weighed and stored (−20°C) until required for subsampling and assay.

Post-mortem samples

At the time of slaughter the animals were stunned using a captive bolt pistol and exsanguinated by severing the major blood vessels of the neck. They were shorn leaving approx. 0·5 cm of wool and the weight of the fleece recorded.

The animals were then dissected and samples of all major organs and tissues taken (approx. 50 samples/sheep) (Table 2). The dissected tissue or organ was inspected for abnormalities, and then weighed and subsampled for analysis. Unexcreted urine in the bladder was collected.

The alimentary tract was ligated at various points in order to separate and collect the contents of its various compartments.

Separate omental, perinephric and subcutaneous fat samples were collected.

Assay of Radioactivity

Scintillator solutions

The stock scintillator solution was 2-(4-t-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole, [butyl-PBD], (0·5% w/v); and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, [DM-POPOP], (0·01% w/v) in toluene. (Solution A.)

The scintillation solution for emulsion counting (solution B) consisted of 2 volumes of solution A and 1 volume of Triton X-100 (Patterson and Greene 1965). Other solutions used were Insta-Gel and Monophase-40 Plus (United Technologies, Packard).

Counting procedure

Tritium measurement was by Beta Liquid Scintillation Counting using either a 1217 Rackbeta (LKB, Sweden) or a Minaxi (Packard, U.S.A.), on auto ^{3}H setting according to the standard protocols in their respective instruction manuals. Results were determined as disintegrations min^{-1} using a quench curve obtained by spiking identical, non-radioactive samples with ^{3}H-Hexadecane.

Each sample was prepared in duplicate and each counted three times sequentially.

Two types of samples were involved: (i) liquid and (ii) solid.

(i) *Tritium assay of liquid samples*

(*a*) *Plasma:* Plasma was counted as an emulsion (0·5 ml plasma and 10 ml Insta-Gel).

Plasma samples (especially those haemolyzed) were persistently chemiluminescent and could only be counted using luminescence correction.

(*b*) *Urine:* 0·5 ml urine + 1 ml water + 15 ml solution B.

Selected urine samples were lyophilized to check for volatile ^{3}H as part of the *in vivo* validation of the label.

The dose standards were also assayed using solution B.

(ii) *Tritium assay of solid samples*

Since the interest of the experiment was in residues, all tissues/organs were lyophilized to constant weight in a Modulo refrigerated unit equipped with a Pirani 10 vacuum gauge (Edwards, Crawley, U.K.). The lyophilized water was sampled to determine volatile ^3H.

Tissue 'solvents' proved totally unsuitable for the preparation of the samples for liquid scintillation counting due to the small sample size possible, and due also to persistent and variable chemiluminescence of the order of 10^5 cpm which the counter's corrector could not handle with the required precision. Combustion of the samples to tritiated water in a Packard 306 Oxidiser (Packard, U.S.A.) was the only viable alternative. This equipment was operated according to the standard protocol (Packard 306 Instrument Manual) in which the freeze-dried sample (150 mg) was combusted in oxygen, purged with steam, and the resultant water injected into a scintillator vial together with Monophase-40 Plus scintillator. Cycle time was 2 min per sample. Recovery, checked by tritium spiking of freeze-dried liver, was better than 95%. Tritium carryover from sample to sample (memory effect) checked with non-spiked freeze-dried liver was 0·5% after one cycle and 0·05% after two cycles. Selected samples were also combusted manually by the modified Schoniger method (Kalberer and Rutschman 1961).

Samples were taken throughout the tissue e.g. with a cork borer, and all tissues were assayed at least in triplicate. Gut contents were homogenized before sampling.

The contents of the faeces sample bag were mixed and 2 × 100 g subsamples were lyophilized (2d) and the dry matter calculated. The dried samples were powdered in a Braun Omnimix and 100 mg were taken in triplicate from each subsample and combusted in the 306 Oxidizer with the ^3H_2O absorbed in 12 ml of Monophase-40 Plus.

A subsample of raw wool was washed with cold Shell X-4 solvent, and the solvent evaporated to yield wax. Wool and wax samples were unsuitable for the 306 oxidiser and were combusted by the Schoniger method.

Fat samples were also unsuitable for the 306 Oxidiser and 200 mg duplicates were dissolved in 20 ml of scintillator A and counted.

Radioimmunoassay of EGF in Blood and Urine

Blood and urine samples were assayed in triplicate and duplicate respectively using the method of Panaretto *et al.* 1982. Standards containing unlabelled rm-α-EGF in the range 0·043–5·54 ng/tube were used and the mixture of ^3H and unlabelled rm-α-EGF used for infusion was iodinated and used as the radioligand. Coefficients of variation for samples were <9%. The antiserum used in these assays was prepared in rabbits against mEGF and was not reactive with human transforming growth factor (Bachem Inc., Torrance, CA) and human EGF (urogastrone, Chiron Corp. CA) nor with rat EGF since no immunoreactivity was detected in rat plasma. The quantities of immunoreactive EGF excreted in urine were obtained from the product of the concentration of EGF (ng ml^{-1}) and excreted volume (ml).

Results

Preparation of Tritiated rm-α-EGF

The material recovered from HPLC of the initial rm-EGF showed a major peak (rm-α-EGF, 80% by weight) and an adjoining shoulder (20% by weight). After tritiation the product was lyophilized and separated twice on HPLC. The yield of tritiated rm-α-EGF was 70 mg with specific activity of 0·52 MBq mg^{-1} (14 μCi mg^{-1}). A sample of this product was rerun on a semi-preparative HPLC column and Fig. 1 shows that the u.v. and radioactive peaks coincide. Labile tritium in the product was less than 0·2%.

Characterization of Tritiated rm-α-EGF

FABMS showed a molecular weight of 6040 (O'Keefe *et al.* 1984; O'Keefe and Sharry 1986); HFPMR produced spectra in agreement with the published values for which amino acid assignments have been made and with those previously reported (Mayo 1984; Mayo *et al.* 1986); u.v. gave an absorbtivity $\{E^{1\%}_{1\,cm}\}$ of 22·6 at 277 nm; and polarimetry an optical activity $\{\alpha^{22°C}_{578\,nm}\}$ of −93°.

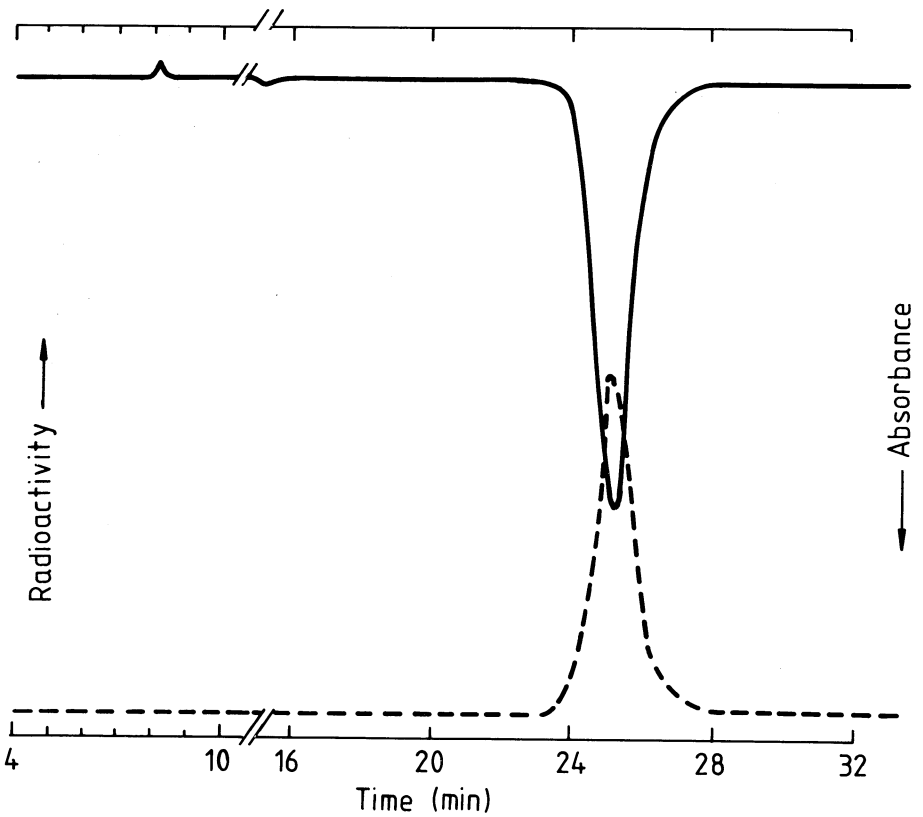

Fig. 1. RP IP HPLC of ^{3}H-rm-α-EGF on a C-18 μBondapak column (30 × 0·8 cm). Mobile phase was acetonitrile-water (26 : 74), 0·04 M in triethylamine acetate, pH 5·6, flow rate 1·5 ml min^{-1}. UV detection at 254 nm, 0·05 AUFS (———). Radioactive detection by LSC, dpm ml^{-1} × 10^{-4} (----).

The amino acid sequence for rm-α-EGF was found to be identical to the published sequence for mEGF (Savage *et al.* 1972). Cysteine-bridging positions 6–20, 14–31 and 33–42 were not determined by this sequencing method. The amino acid analysis was consistent with the test material having the structure of EGF (Savage and Cohen 1972). Tryptophan was not determined since it is destroyed during acid hydrolysis. These results corroborate the hypothesis that the 'α' peak is EGF.

Retention of biological activity for the rm-α-EGF was demonstrated by a positive response to two determinants: (1) The receptor binding affinity of rm-α-EGF, ^{3}H-rm-α-EGF and mEGF were equivalent. (2) There was complete inhibition of wool growth after 8 d as shown by fleece shedding. This was the expected quantitative dose-response predicted from numerous defleecing experiments on the sheep using mEGF, rm-EGF and rm-α-EGF (Panaretto *et al.* 1982; Allen *et al.* 1987; J. W. Bennett, L. F. Sharry, and J. H. O'Keefe, unpublished data).

Tritium Levels of in vivo *Samples*

Plasma

The results presented in Fig. 2 show that there was still ^{3}H circulating in the blood after 8 d. Maximum levels were obtained about 24 h after the beginning of the infusion and were between 2 and 2·6% of the ^{3}H dose per litre of plasma. If all this radioactivity was

circulating as tritiated EGF, the blood level maxima would be in the range 84–109 ng ml^{-1}. Unlike the EGF levels measured by RIA the ^3H levels did not fall to zero after the infusion was completed.

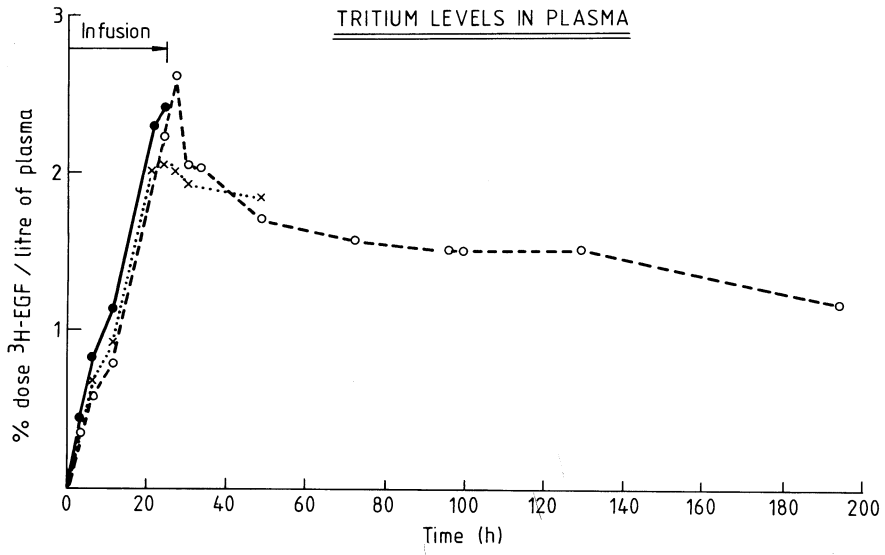

Fig. 2. Tritium in blood plasma during and after intravenous infusion (24 h) of ^3H-rm-α-EGF [4·2 mg; 1·554 MBq (42 μCi)]. The results are for the means of three sheep slaughtered 24 h (●——●), 48 h (×···×) post-infusion start, and for one sheep slaughtered 192 h (○– – –○) post-infusion start.

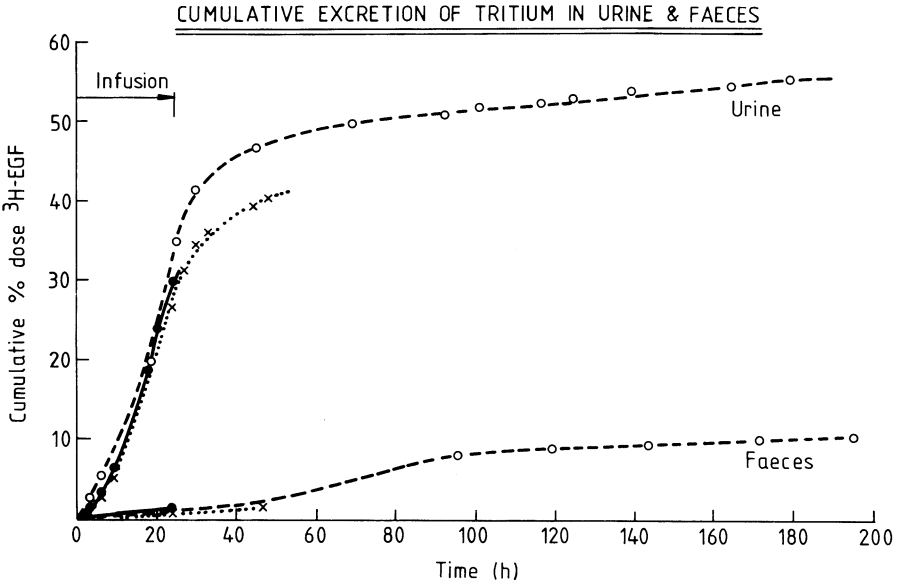

Fig. 3. The cumulative recovery of tritium in urine and faeces during and after intravenous infusion (24 h) of ^3H-rm-α-EGF. The results are for the mean of three sheep slaughtered 24 h (●——●), 48 h (×···×) post-infusion start, and for one sheep slaughtered 192 h (○– – –○) post-infusion start.

Urine and faeces

Fig. 3 shows the cumulative urine and faeces excretion for the three groups of sheep. Most of the tritium was excreted in the urine averaging 30% at 24 h to 40% at 48 h and 55% at 192 h (Fig. 4). Tritium was still being excreted after 8 d.

Table 1 shows the proportion of stable and labile ^3H in the urine. Labile ^3H values were 3·7% (6 h) and 8·4% (30 h) while stable ^3H values were 91·9% and 88·3% respectively.

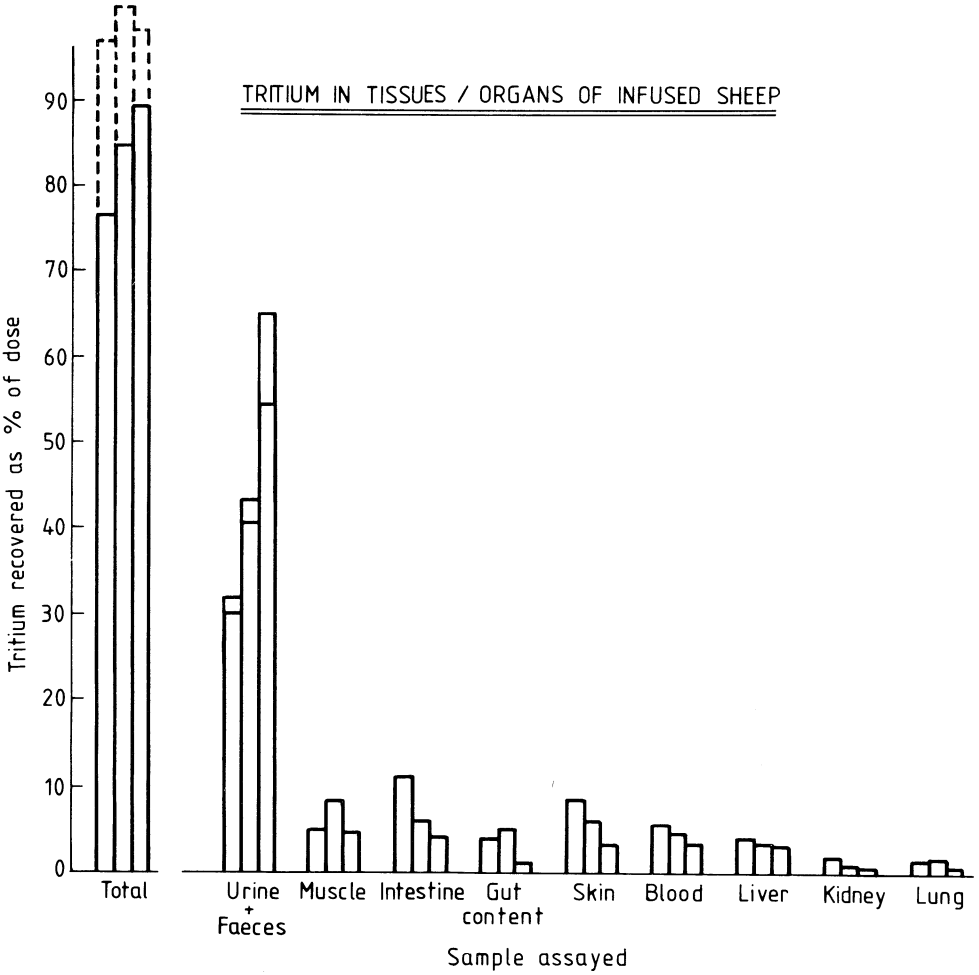

Fig. 4. Total tritium recovery and major locations of tritium recovered from sheep slaughtered after a 24 h infusion of ^3H-rm-α-EGF. In each instance recoveries at 24, 48 and 192 h after the start of infusion are arranged sequentially from left to right. Total ^3H recovered was 97·4% (24 h), 100·5% (48 h), and 97·8% (192 h). Stable ^3H (solid); labile (dotted). The recovery from excretion products (urine + faeces) was 31·6% (24 h), 42·5% (48 h) and 65·1% (192 h).

Tritium Levels of Tissue Samples

Table 2 shows the recovery, ranked in three groups, of ^3H 48 h after the start of the infusion in three sheep from all organs sampled. The organs/tissues that made a major contribution to the ^3H recovery are shown in Fig. 4.

With time the percentage of ^3H in the gastrointestinal tract, the skin, the blood and kidney decreased; the percentage in the liver remained steady; and the percentage in the faeces increased. There was no ^3H in the wool sampled.

Table 1. Tritium in urine (stable and labile)[A] from seven sheep infused (i.v. 24 h) with ^{3}H-rm-α-EGF (120 μg kg^{-1})

Time[B] (h)	Stable (%)	Labile (%)	Total
6	91·9	3·7	95·6
9	93·2	3·3	96·5
24	93·4	5·8	99·2
30	88·3	8·4	96·7

[A] For monitoring of stability of ^{3}H label.
[B] Time from start of infusion.

There were no detectable differences from either fat location (omental, perinephric or subcutaneous) or slaughter time on fat ^{3}H content. The values averaged 1·85 Bq g^{-1} (50 pCi g^{-1}) equivalent to 5 μg kg^{-1} assuming all tritium is present as EGF. Fat results were expressed as pCi g^{-1} since the actual total *in vivo* weights were not determined. Thus, assuming the sheep carcass is 20% fat (Berg and Butterfield 1976), ^{3}H from fat accounted for approximately 0·8% of the administered dose.

Table 2. Distribution of ^{3}H in the sheep at 48 h

I.V. infusion for 24 h, 0·16 MBq (4·2 μCi), 4·2 mg, 3 sheep. Tritium in 8 main organs/tissues 36·8% (Fig. 4); tritium in remaining locations 2·8%; total tritium recovered 100·5% of dose

Contributions >0·5% of administered ^{3}H-EGF		
Gut contents	Caecum	Small Intestine
	Large Intestine	Rumen
Intestines	Abomasum	Omasum
	Large Intestine	Reticulum
	Small Intestine	Rumen
Others	Blood	Mid-side skin
	Kidneys	Inguinal skin
	Liver	L. Dorsi muscle
	Lungs	Gluteal muscle
	Fat	Omental
		Perinephric
		Subcutaneous
Contributions 0·1 to 0·5% of administered ^{3}H-EGF		
	Abomasum content	Heart
	Colon content	Spleen
	Oesophagus	Tongue
		Brain
		Parotid Gland
Contributions <0·1% of administered ^{3}H-EGF		
Gland	Adrenal	Pancreas
	Pituitary	Trachea
	Pineal	Gall bladder
	Sublingual	Gall bladder content
	Submaxillary	Urinary bladder
	Thyroid	Metatarsal bone
	Larynx	Metatarsal marrow
		Wool

There was no detectable difference between L-dorsi and gluteal muscles (670 pCi g^{-1}; 24·8 Bq g^{-1}; 67 μg kg^{-1}). Assuming the sheep carcass is 16% muscle (Berg and Butterfield 1976), ^{3}H recovered from muscle was 5·5% (1 d), 8·3% (2 d) and 4·5% (8 d) of the administered dose.

Kidney showed the highest tissue concentration of EGF residues with 740 μg kg^{-1} (7·40 nCi g^{-1}, 274 Bq g^{-1}) after 1 d and 172 μg kg^{-1} (1·72 nCi g^{-1}, 64 Bq g^{-1}) after 8 d.

Levels of EGF and Its Metabolites in Edible Sheep Meat

The Australian Bureau of Statistics (1985/6) estimated the annual *per capita* consumption of sheep meat and offal as 23·7 kg and 3·3 kg respectively. Of this 17·1 kg of meat and 2·38 kg of offal was derived from fat lambs, not shorn prior to slaughter, and therefore not treated with EGF. Mutton (ewes, wethers, rams) thus accounts for 6·6 kg of meat and 0·92 kg of offal annually *per capita*. Assuming all these sheep are treated with EGF at a dose rate of 120 μg kg^{-1} live body weight and that meat is 80% muscle and 20% fat, the amount of EGF may be calculated from the residue data. Kidney had the highest EGF concentrations and was selected for offal calculations. All times used were administration times prior to slaughter.

Muscle, with EGF residues of 38·5 μg kg^{-1} after 1 d and 32·0 μg kg^{-1} after 8 d, would result in an average daily ingestion in meat of 0·6 μg and 0·5 μg of EGF respectively. Fat, with a residue of 5 μg kg^{-1} after both 1 and 8 d, would result in the negligible ingestion of $<0·02$ μg. The residues in offal of 740 μg after 1 d and 172 μg after 8 d would result in an average daily ingestion of 2·0 μg and 0·5 μg respectively. The total average daily ingestion of EGF from meat and offal is thus 2·6 μg and 1·0 μg when administered 1 and 8 d prior to slaughter respectively.

Table 3. Cumulative urine levels of EGF (by RIA) and tritium[A] (by LSC) in sheep dosed with ^{3}H-rm-α-EGF

Time from start of infusion (d)	EGF dose (mg)	Immunoreactive (RIA) EGF in Urine (mg)			% Dose (0–24 h)		Ratio RIA Tritium[B]
		0–24 h	24–48 h	Total	RIA	Tritium	
1 (3 sheep)	4·37	0·19		0·19	4·4	21·5	0·20
	3·39	0·32		0·32	9·3	32·5	0·29
	4·09	0·54		0·54	13·2	36·4	0·36
2 (3 sheep)	4·20	0·25	0·13	0·38	6·0	29·0	0·21
	3·99	0·32	0·08	0·40	8·0	27·0	0·30
	4·54	0·38	0·24	0·62	8·4	23·0	0·37
8 (1 sheep)	3·97	0·22	0·30	0·52	5·5	35·0	0·16

[A] Assuming all tritium as ^{3}H-rm-α-EGF. [B] A measure of the metabolized rm-α-EGF.

RIA Levels of rm-α-EGF in Plasma and Urine

No immunoreactive EGF was detected in pre-infusion samples of plasma or urine.

Plasma concentration of rm-α-EGF rose gradually during infusion reaching a mean ± s.e. concentration of 24·7 ± 1·4 ng ml^{-1} (n = 13) during the last 4 h of infusion. Concentrations fell quickly 1·5–3 h after infusion finished reaching levels of 5·4 ± 1·6 ng ml^{-1} respectively. Concentrations detected 4·5–6 h after infusion finished were <1 ng ml^{-1} and no EGF was detected in plasma 24 h after infusion started.

There was no discernible effect of EGF on urinary volume, and the quantities voided and the recoveries of immunoreactive EGF as a percentage of the dose are included in Table 3.

Levels of Tritium and rm-α-EGF in Plasma and Urine

The radioassay estimated total ^{3}H circulating and the radioimmunoassay, the level of rm-α-EGF.

The concentration of rm-α-EGF (ng ml^{-1}) in the plasma and urine (Table 3) was calculated both by the RIA procedure and the tritiated EGF assay (assuming that all tritium was present as ^{3}H-EGF). At 24 h after the start of infusion the mean ration RIA/^{3}H for the seven sheep was 0·27 for both plasma and urine and the ratio was relatively constant over the infusion period for both. If the RIA was a true indication of the level of rm-α-EGF then about 25–30% of the ^{3}H-EGF dose was circulating as EGF and the remainder had been metabolized.

Discussion

Choice of Isotope and Validation of the Labelled Compound

^{125}I-EGF studies have been reported in the rat (Thornburg *et al.* 1984, 1987) using EGF iodinated by the standard chloramine T procedure (Hunter and Greenwood 1962) but the iodinated product was not purified and not characterized, though this labelling process is known to produce a mixture partially separable by HPLC (Magun *et al.* 1982; Matrisian *et al.* 1985). Since there is no known way of unequivocally characterizing the small (μg) amounts of the product of the iodination process, it has not been established which if any of the HPLC peaks is ^{125}I-EGF. Also, the ^{125}I is attached to tyrosine only, and of the 5 tyrosines, those which are in exposed positions on the folded EGF molecule would presumably be preferentially labelled as has been suggested for similar molecules containing hindered tyrosines (Bolton 1985). ^{125}I is thus tracing tyrosine only and the degree of tracing is dependent on its position in the molecule. The ^{125}I-EGF is *non-isotopically* labelled with the large iodine atom and considerable validation of the material as a tracer for EGF would seem mandatory, but we have seen no report of this.

An isotopically labelled compound was essential to our study. However, without synthesis of the EGF molecule which has only recently been reported, and then only in small quantities (Heath and Merrifield 1986), ^{3}H labelling of the intact molecule was the only feasible method. This use of ^{3}H as a tracer is in keeping with recent trends since studies using HPLC and ^{3}H-NMR confirm that ^{3}H is an efficacious label (Williams *et al.* 1988). For the project envisaged, a generally labelled compound with stable isotope incorporation was required, and considerable experimental effort was devoted to the validation of the labelling procedure, *in vivo* and *in vitro*.

The product of the labelling process was validated by HPLC separation and extensive physicochemical characterization (FABMS, HFPMR, u.v. spectrophotometry, optical activity, amino acid analysis and sequencing). This extensive testing indicated that the product was ^{3}H labelled rm-α-EGF i.e. the product was identical to the rm-α-EGF starting material with the exception of isotopic hydrogen (^{3}H) substitution.

Retention of biological activity was demonstrated by radioreceptor assays in which rm-α-EGF, ^{3}H-rm-α-EGF and mEGF were found to be equally biopotent. Previous batches from the tritiation process were tested in the mouse for precocious eyelid opening (Cohen 1962; Allen *et al.* 1987). This bioassay showed that ^{3}H-rm-α-EGF and mEGF were equally potent. Major evidence for quantitative retention of the biological activity of interest to this project was the complete inhibition of wool growth as shown by fleece shedding after 8 d which followed the expected dose-response pattern. Extensive work on EGF fragments has shown that defleecing activity in the sheep is critically dependent on compound structure and this quantitative retention of defleecing activity is advanced here as presumptive evidence that this aspect at least of the EGF structure is unchanged. The monitoring of urine sampled by lyophilization confirmed that the tritium was stably bound to the ^{3}H-rm-α-EGF.

Tritium Levels in Organs and Tissues

The distribution of stable ^{3}H-EGF in tissues and organs, particularly those important in the human diet, was determined for residue studies. For this reason all tissues/organs were freeze-dried to yield stable ^{3}H before assay. A correction was applied for volatile ^{3}H, mainly ^3H_2O, by assay of the lyophilisate. Volatiles such as water, ammonia and methane together with non-volatiles such as amino and fatty acids are known products of the degradation of peptides in the rumen (Cotta and Hespell 1984).

There was rapid excretion of EGF and its metabolites in urine and faeces: 31·6% at 24 h, 42·5% at 48 h, and 65·1% at 192 h. However, significant amounts were retained in body tissues. As shown in Fig. 4 most of the tritium retained in tissues was located in eight organs. These accounted for 41·9% after 1 d with only 3·1% for the remaining tissues/organs, 36·8% after 2 d with 2·8% for the remaining tissues/organs, and 22·1% after 8 d leaving 1·8% for the remaining tissues.

Virtually complete recovery of the ^{3}H labelled EGF administered was achieved: 97·4% after 1 d, 100·5% after 2 d, and 97·8% after 8 d. The corresponding values for stable ^{3}H were: 77·4% (1 d), 85·5% (2 d), and 89·9% (8 d). These recoveries validate the sampling and assay procedures used.

The persistence of ^{3}H in plasma could be explained by the incorporation of labelled degradation products from the rumen (particularly amino and fatty acids) into other peptides and proteins. Supporting this hypothesis is the disappearance of immunoreactive EGF from the urine and plasma within 24 hours of cessation of infusion. Further studies with ^{3}H-EGF are thus needed to definitively establish the metabolic fate of EGF *in vivo*. In particular, it would be of considerable interest to determine the structure of metabolic fragments. Such information is essential in the formulation of a high-grade hypothesis on the mode of action of EGF which would have relevance to other polypeptides such as the therapeutically important insulin.

EGF and Its Metabolites in the Food Chain

Though the present study detected no abnormalities in the tissues and organs during dissection (B. A. Panaretto), EGF has been shown by *in vitro* and *in vivo* studies to be a mitogen (King and Carpenter 1983). The data from the residue studies were therefore used to calculate the amounts of EGF and its metabolites likely to be ingested in meat from treated sheep.

These calculations showed that the average daily intake of EGF and metabolites would be 2·6 μg from animals treated 1 day prior to slaughter and 1·0 μg from animals treated 8 days previously. Assuming that this small quantity of ingested EGF would be attacked in the gut by the pancreatic enzymes trypsin and chymotrypsin (Savage *et al.* 1972) it was concluded, tentatively pending long term studies specifically aimed at this aspect, that no harmful effects were likely from the consumption of meat and offal from sheep treated with EGF at the level reported herein.

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