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(54) Title: IMPROVEMENTS IN OR RELATING TO THE USE OF EPIDERMAL GROWTH FACTOR

(57) Abstract

A method for the promotion of growth, particularly in the young of the pig and other farm animals, comprises administering epidermal growth factor (EGF) and/or dexamethasone or a physiologically active derivative thereof. EGF is also of value for the manufacture of a medicament for use in veterinary and human medicine in effecting an increase in the level of iron in a patient's bloodstream. A composition comprising EGF and an iron-providing material is of value both for the animal husbandry and the medical use of EGF.

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IMPROVEMENTS IN OR RELATING TO THE USE OF

EPIDERMAL GROWTH FACTOR

This invention relates to the use of epidermal growth factor (EGF) in animal husbandry and in veterinary and human medicine.

It has been found that novel compositions comprising epidermal growth factor and an iron-providing material are of value both in animal husbandry and in veterinary and human medicine.

In its first aspect, therefore, the present invention relates to the promotion of growth, particularly in farm animals such as piglets.

The majority of farm animals differ from humans in being unable to take up immunoglobulins across the placenta and instead achieve a measure of immunity from their mothers by the uptake of antibodies from the mother's milk through a "leaky", immature gut cell structure. However, although this uptake is achieved quite rapidly after birth, for example within a period of about 48 hours in the piglet, the replacement of the immature gut cells with mature cells can then take a considerable time, for example up to four weeks in the piglet. This period which exists before a fully mature cell structure is developed can create various problems in animal husbandry and in particular prevents the utilisation of adult-type diets, making the neonatal animal dependent on suckling for its essential nutrients. The extended weaning period also means that the mother animal, for example the sow, cannot be returned to breeding as rapidly as commercial considerations would dictate.

A number of academic studies have been undertaken upon the effect of hormones and other compounds on the development of epithelial cells. Thus, a combined tri-iodothyronine/prednisolone treatment has been shown to induce precocious maturation of some digestive enzymes in the suckling pig (Baintner and Németh, Arch. Tierernährung, 1982, 32, 229-234). However, the majority of the reported studies, including those utilising epidermal growth
factor reported by Malo and Ménard, Gastroenterology, 1982, 83, 28-35, by Oka et al, Endocrinology, 1983, 112, 940-944 and by O'Loughlin et al, American Journal of Physiology, 1985, 249 (Gastrointestinal Liver Physiology 12), 6674-6678, have been carried out with laboratory animals and cannot be extrapolated to farm animals such as pigs, rats and mice in particular differing quite significantly from farm animals in their epithelial cell behaviour. Moreover, to be of commercial value a treatment must increase certain selected enzyme activities without causing additional enzymic or other changes of an undesirable nature and certain of the treatments which have been reported in laboratory animals have led to the precocious maturation of some digestive enzymes but with an accompanying reduction in the overall growth of the animal.

It is an object of the present invention to provide a method for achieving an appropriate spectrum of precocious maturation of digestive enzymes in farm animals, and in particular in piglets, and thereby to enable the promotion of growth therein.

According to the present invention a method for the modification of the digestive system of the young of the pig and other farm animals comprises administering epidermal growth factor to a young animal having an immature gut cell structure.

Since the invention is of particular interest in relation to the piglet it will for simplicity be described hereinafter primarily in relation to that animal.

It has been found that, in the piglet, epidermal growth factor (EGF) beneficially enhances both sucrase and maltase activity but without any marked effect upon the activities of lactase and alkaline phosphatase. This spectrum is particularly advantageous since sucrase and maltase degrade unassimilable sucrose and maltose into assimilable glucose and enhancement of such activity is therefore beneficial, whilst lactase is required for assimilation of the lactose the piglet obtains from the sow's milk so that avoidance of any serious level of reduction in such activity is again important.
Although the ability of epidermal growth factor to enhance the energy supply derivable from carbohydrates in the piglet constitutes a surprising and fundamental contribution to pig husbandry, it will be appreciated that this contribution would be increased by the provision of a method of directly enhancing nutrient uptake and improving protein production, this being a second object of the present invention which, coupled with the first, provides a promotion of performance with an improvement of the utilisation and uptake of feed constituents.

According to the present invention a method for the enhancement of nutrient uptake in an animal or human comprises administering thereto dexamethasone or a physiologically active derivative thereof.

Although a number of in vitro studies on amino acid transport in mammalian cells using a variety of compounds have been reported in the literature, for example by Shotwell et al, Biochimica et Biophysica Acta, 1983, 737, 267-284, the results achieved by us with dexamethasone in the piglet indicate the particular value of this compound in the enhancement of nutrient uptake in vivo, particularly in farm animals. The use of dexamethasone will also for simplicity be described hereinafter primarily in relation to the piglet, which may be one having an immature gut cell structure or a somewhat older animal in which the gut cell structure is mature.

It has been found that, in the piglet, dexamethasone beneficially modifies the digestive system through enhancing the uptake of certain amino acids by epithelial cells. The use of dexamethasone will therefore increase protein production and may conveniently be coupled with the use of epidermal growth factor which increases the ability of the immature piglet to break down carbohydrates to supply energy.

The present invention thus further includes a method for the promotion of growth in the young of the pig and other farm animals which comprises administering to a young animal epidermal growth factor and dexamethasone or a physiologically active derivative of dexamethasone.
The EGF used in the present invention may conveniently be derived from various mammalian sources since the active sequence thereof is closely conserved among species. Alternatively, synthetic EGF may be used that may optionally have variations in the molecule, within the active sequence or particularly outside it, which do not correspond to those found in nature or which constitute an admixture of variations found in different species in nature. Essentially any compound expressing the activity of the natural hormone may be used. The term epidermal growth factor is therefore used herein in a general sense to include all such natural materials and their synthetic equivalents, as well as variants thereon retaining the physiological activity of the molecule. Apart from the other animals detailed hereinafter such as cattle and the goat, sheep and horse, sources include the mouse, rat and rabbit, and even human EGF (urogastrone). In principle, EGF of the same species as the recipient is to be preferred in order to avoid the possibility of setting up undesirable side reactions, although the short period of administration of the EGF renders such an occurrence relatively unlikely. Pig EGF is therefore of particular interest and could be produced by genetic engineering as has already been done for mouse EGF, which therefore provides another preferred form of EGF. The terms pig EGF and mouse EGF, for example, are used herein in a general sense once again to include the natural material and its synthetic equivalent, as well as variants thereon retaining the physiological activity of the molecule. The structure of mouse EGF is shown in UK Patent 1,417,776 and examples of possible active variants of the natural structure are also shown therein illustrating the type of variation which may also be applied with EGF derived from other sources whilst retaining activity. The surprising nature of the results obtained in the piglet is illustrated by the fact that mouse EGF produces far better results in the pig than in the mouse.
As explained previously, it is important that the gut cells of the piglet remain in the immature state for a sufficient time to allow the transfer of immunity from the sow to take place. Administration of the EGF is therefore preferably commenced when the piglets are 2, 3 or 4 days old, especially 2 or 3 days, and may conveniently be continued for a period of 2, 3 or 4 days, for example 3 days. Thus, treatment will therefore usually take place during the period from when the piglet is 2 days old to when it is a week old, although it may only be necessary to administer the EGF during a successive 2 or 4 or particularly 3 of these days.

Since EGF has only a short half life in vivo when given parenterally (about 2½ minutes when given intravenously) it is appropriate either to administer a series of doses during the period of treatment, for example at eight hour intervals or, preferably, to administer the EGF in the form of a controlled release preparation (including a pulsed release preparation), for example in a liposome preparation such as is described in UK Patent EP 0007714 or in a polymeric preparation such as is described in UK Patent 2112381, from which the EGF is released during the required time period. Other alternatives for effecting a gradual release of the EGF include implants (polymers and waxes), mineral oil formulations, colloidal suspensions and the use of forms of EGF of low solubility. Although it may be appropriate to use dosages outside the following ranges, for example depending on the species of EGF which is used, dosages are conveniently in the range from 5 or 10 μg/kg of the animal's weight up to as much as 100 μg/kg given at 8 hour intervals, dosages towards the lower end of this range being preferred, however, as being better tolerated whilst still achieving the desired gut maturation, for example dosages in the range of 10 to 30 μg/kg such as 20 μg/kg. For controlled release preparations the release of a similar dosage during an 8 hour period may generally be suitable, subject to any variations upwards or downwards which may be appropriate in view of the altered situation involving gradual release of the same amount but with lower concentration levels existing in vivo at any one time than
are achieved by administration of the single dosage. Both single dosages and delayed release preparations (except implants) are commonly administered parenterally by injection, for example subcutaneously. The EGF is therefore commonly used in the form of a composition comprising a physiologically acceptable diluent or carrier which is sterile and also pyrogen-free, for example in sterile, pyrogen-free physiological saline.

Following the administration of EGF to the animal the administration of a solid, semi-solid or liquid adult-type diet, as opposed to the mother's milk, may conveniently be commenced at the end of the EGF dosage period (either by multiple individual or single controlled release administration) indicated previously. The adult-type diet may, for example, comprise conventional piglet feed materials but it will often be convenient to commence feeding with a liquid diet and then progress through a semi-solid diet to a solid diet. Following such feeding with an adult-type diet an enhanced rate of growth will be achieved in the animal as compared with that observed at the same age when relying upon the type of feeding which is necessary in the absence of the EGF treatment.

The present invention thus includes the use of epidermal growth factor in growth promotion in farm animals. Although the invention is particularly directed to piglets it is also of especial interest for use with cattle, goats, sheep and horses (calves, kids, lambs and foals, like the piglet, all acquiring passive immunity at birth through an immature gut).

Dexamethasone (9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione) can be used as such or in the form of a physiologically active derivative such as a physiologically acceptable ester, which often acts by the release of dexamethasone in vivo, for example the 21-acetate, 21-(3,3-dimethylbutyrate), 21-phosphate, 21-phosphate disodium salt, 21-tetrahydrophthalate, 21-dimethylaminoacetate, 21-isonicotinate, 21-phenylpropionate, etc. The dexamethasone (or its derivative) may conveniently be administered over the same time period as has been described herein for EGF. Although dexamethasone does not suffer from the
same short half life in vivo as does EGF it may still be
convenient to present this compound also in controlled release
form, for example as described for EGF or through the formulation
of a derivative thereof such as is employed in preparations such
as DEXAFORT (Intervet Trade Mark - contains the phenylpropionate
and sodium phosphate derivatives). Dosage levels are in general
somewhat higher in relation to the animal's weight than the doses
of EGF quoted previously, an eight hourly dosage in the range
of 50 or 100 μg/kg up to as much as 400 μg/kg being suitable,
particularly 100 to 200 μg/kg. Controlled release preparations
may conveniently release a similar dosage during an 8 hour period,
subject to the same provisos as made for EGF, for example a
controlled release of 1 or 2 mg/kg over 3 days being suitable.
The modes of administration and formulation of the dexamethasone
may conveniently be similar to those described previously for EGF
although oral administration may be of greater interest in the
case of dexamethasone.

Dexamethasone does, however, also have potential interest for
use at later stages (including adulthood) in the life of animals,
particularly those mammals described hereinbefore, for the
enhancement of nutrient uptake. In such a context its
incorporation into an animal, or even a human, foodstuff provides
an alternative mode of administration.

Feeding with an adult-type diet may be commenced following
dexamethasone treatment as described for EGF. The present
invention thus includes the use of dexamethasone in growth
promotion in farm animals. Once again, although the invention is
particularly directed to piglets it is also of interest for use
with other animals, particularly farm animals such as cattle,
goats, sheep and horses.

As indicated previously, a preferred method according to the
present invention utilises both EGF and dexamethasone. The
present invention thus includes a product comprising epidermal
growth factor and dexamethasone or a physiologically active
derivative of dexamethasone for simultaneous, separate or
sequential use in growth promotion. Particular ways of presenting the two compounds include a composition for use in growth promotion comprising epidermal growth factor and dexamethasone or a physiologically active derivative of dexamethasone together with a physiologically acceptable diluent or carrier and a kit for use in growth promotion comprising in association epidermal growth factor and dexamethasone or a physiologically active derivative of dexamethasone. The proportions of the two compounds in such a composition or kit will be appropriate to provide dosages in the ranges discussed hereinbefore, the amount of dexamethasone or its derivative therein usually being greater than that of EGF, and formulation of the compounds together in the composition or individually in the kit may conveniently be as described previously. In the use of both composition and kit the compounds may be presented in unit dosage form, i.e. in portions containing a unit dose or a multiple or subunit thereof.

It will be appreciated that it is also possible to utilise EGF with an alternative enhancer of nutrient uptake than dexamethasone and to utilise dexamethasone with an alternative modifier of immature gut cell structure than EGF. Moreover, where desired it is possible to use additional materials of value specifically in growth promotion or in maintaining the general well being of the animal together either with EGF or dexamethasone or its derivative alone or with EGF and dexamethasone or its derivative used together. Where appropriate, such additional materials may be incorporated into compositions or kits containing EGF and/or dexamethasone or its derivative. Examples relating specifically to growth promotion include tri-iodothyronine (T3), its precursor thyroxine (T4) and analogues of T3 and T4, insulin, glucagon and also growth hormone (various species types of growth hormone and insulin being utilisable as described for EGF). Examples relating to maintenance of general well being include various iron-providing materials, for example those which are the subject of UK Patents and Patent Applications 2117766B, 2136806A, 2157686A and particularly 2128998B. It is common practice with piglets to
administer an iron supplement at about 2 days of age and the EGF and/or dexamethasone or its derivative may conveniently be administered at the same time as this supplement, either in one composition or separately.

Although the invention has been described primarily with reference to piglets the mode of application to the other animals mentioned herein is essentially similar with dosages of EGF and dexamethasone being at the same µg/kg level. In some cases, however, the uptake of immunoglobulins through the immature gut cell structure can continue for somewhat longer than in the piglet so that it may be convenient to delay the commencement of treatment, for example to an age of from 5 to 10 days.

In its first aspect, the invention is illustrated by Examples 1, 2 and 3 which follow hereinafter.

In its second aspect the present invention relates to the treatment of iron deficiency and in particular to pharmaceutical compositions and foodstuffs containing iron.

An adequate supply of iron to the body is an essential requirement for tissue growth in both man and animals. Although there is normally an ample amount of iron in the diet, the level of absorption of iron from food is generally low so that the supply of iron to the body can easily become critical under a variety of conditions. Iron deficiency anaemia is commonly encountered in pregnancy and may also present a problem in the newly born, particularly in certain animal species such as the pig. Moreover, in certain pathological conditions there is a malabsorption or maldistribution of body iron leading to a state of chronic anaemia. Such malabsorption or maldistribution is seen in certain intestinal disorders and in chronic diseases such as rheumatoid arthritis, certain haemolytic diseases and cancer.

Although a wide range of iron compounds is marketed for the treatment of iron deficiencies and the results thereof, and for the prophylaxis of such iron-deficiency states, the level of iron uptake by the body from these compounds is often quite low thereby necessitating the administration of relatively high dosage levels.
of the compound. The administration of high dose, poorly absorbed, iron complexes may cause siderosis of the gut wall and a variety of side effects such as nausea, vomiting, constipation and heavy malodorous stools.

It is therefore an object of the present invention to provide a means of enhancing the uptake of iron and it has been found that this may be achieved through the use of epidermal growth factor (EGF), usually through the incorporation into a pharmaceutical composition or a foodstuff of EGF. Although therapeutic applications of EGF have previously been described, for example in UK Patent 1,417,776 which describes the use of EGF in inhibition of the secretion of acidic gastric juice, there has never previously been any indication that EGF had a role in the enhancement of iron uptake by the body.

Accordingly the present invention comprises the use of epidermal growth factor for the manufacture of a medicament for use in effecting an increase in the level of iron in a patient's bloodstream.

The present invention extends to the use of EGF to enhance iron uptake from a wide variety of iron-providing materials, which may contain iron in the ferrous or particularly the ferric form, in both humans, animals and birds. Particular interest centres on the treatment of mammals, especially humans and also pigs, for example piglets.

The iron-providing material may be any physiologically acceptable substance capable of raising the level of iron in the bloodstream on administration in vivo, including both iron salts and iron complexes. Examples of specific iron-providing materials include, particularly for human use, ferric chloride, ferric ascorbate, ferric citrate, ferrous fumarate, ferrous gluconate and ferrous succinate, and, particularly for use in piglets, compounds, including some of those mentioned above, which are described in UK Patent 1,322,102 and US Patent 4,362,710, for example iron (ferric) dextran, ferrous fumarate and ferric citrate. Also of particular interest for both human and animal
use are the iron complexes which are the subject of UK Patents and Patent Applications 2117766B, 2136806A, 2157686A and particularly 2128998B, especially (3-hydroxy-2-methyl-4-pyrone)3 iron(III) and related homogeneous and heterogeneous 3:1 hydroxypyrone:iron(III) complexes.

The EGF may be administered alone in order to enhance iron uptake from either normally ingested or specifically administered iron-providing materials and in the latter aspect the present invention therefore includes a product comprising epidermal growth factor and an iron-providing material for simultaneous, separate or sequential use in growth promotion, for example a kit comprising the two components in association. However, it will most usually be formulated together with an iron-providing material and the present invention therefore includes a product comprising an iron-providing material and epidermal growth factor for use in therapy.

The EGF used in the present invention may conveniently be derived from various natural sources, particularly mammalian sources, since the active sequence thereof is closely conserved among species. Alternatively, synthetic EGF may be used that may optionally have variations in the molecule, within the active sequence or particularly outside it, which do not correspond to those found in nature or which constitute an admixture of variations found in different species in nature. Essentially any compound expressing the activity of the natural hormone may be used. Accordingly, when describing this second aspect the term epidermal growth factor is therefore again used herein in a general sense to include all such natural materials and their synthetic equivalents, as well as variants thereon retaining the physiological activity of the molecule. Specific sources include mouse, rat, rabbit, cattle, goat, sheep, horse, pig and human EGF (urogastrone). In principle, EGF of the same species as the recipient is to be preferred and pig and human EGF are therefore of particular interest. Also of some especial interest is mouse EGF which has been produced by genetic engineering, although this
technique could also be applied to the production of pig and human EGF, etc, the terms pig EGF and mouse EGF, for example, being used in a general sense as mentioned previously. Also as mentioned previously, the structure of mouse EGF is shown in UK Patent 1,417,776 and examples of possible active variants of the natural structure are also shown therein illustrating the type of variation which may also be applied with EGF derived from other sources whilst retaining activity.

EGF may be used according to the present invention for the manufacture of medicaments having a variety of forms. Usually, however, these will comprise, in addition to the EGF and an iron-providing material, a physiologically acceptable diluent or carrier. The present invention therefore includes a pharmaceutical composition comprising an iron-providing material, epidermal growth factor and a physiologically acceptable diluent or carrier.

The iron-providing material and the EGF may be formulated together in a pharmaceutical composition by a variety of methods. For instance, they may be applied as an aqueous, oily or emulsified composition incorporating a liquid diluent which may often be employed in injectable form for parenteral administration and therefore may conveniently be sterile and pyrogen free. For certain other uses a diluent which is sterile but not necessarily pyrogen free may be appropriate. As regards liquid diluents or carriers therefore, there is often particular interest in those which are sterile. Oral administration is often preferred for the treatment of iron deficiency anaemia in humans and the present invention is suited to such a route of administration. Although compositions incorporating a liquid diluent may be used for oral administration, it is more usual, at least in humans, to use compositions incorporating a solid carrier, for example a conventional solid carrier material such as starch, lactose, dextrin or magnesium stearate. Such solid compositions may conveniently be of a formed type, for example as tablets, capsules (including spansules), etc.
As indicated, liquid compositions are of particular interest in relation to parenteral administration, a requirement for which arises with humans in certain contexts but also particularly in a veterinary context, for example with pigs. The problems of iron deficiency anaemia in newly born pigs arise primarily during the first three weeks or so of their life when a very rapid weight gain takes place. The usual routes for administration of iron-providing materials to young piglets in the context of the present invention are parenteral, for example intramuscular, or oral, for example as a liquid preparation "injected" into the mouth. However, an alternative approach is to enhance the iron content of the milk on which the piglets are feeding by treating the mother pig using oral or parenteral administration, for example with an injectable slow release preparation (such an approach may also be of interest in a human context). As indicated previously, slow release preparations for the parenteral administration of EGF are of particular interest in view of the short half life of EGF in vivo when given by such a route (about 2½ minutes when given intravenously).

Other forms of administration than by injection or through the oral route may also be considered in both human and veterinary contexts, for example the use of suppositories or pessaries, or of compositions for buccal or nasal administration. Further details regarding the formulation of iron-providing materials are to be found in the patents relating to such compounds mentioned hereinbefore.

The compositions may be formulated in unit dosage form, i.e. in the form of discrete portions containing a unit dose, or a multiple or sub-unit of a unit dose. The dosage of the iron-providing material will of course depend on the particular material which is used but it may be indicated by way of guidance that the daily requirement of iron for the adult human is generally regarded as being from 2 to 4 mg and that the dosage is therefore that which is appropriate to ensure this level of
intake. Further information on the dosage levels of the iron compounds is to be found in the ABPI Data Sheet Compendium published annually by Datapharm Publications Ltd., London, U.K., and in the various patents and patent applications mentioned hereinbefore. As regards the EGF, dosage will again depend on the particular iron-providing material to be used in conjunction with the EGF but, as a guide, it may be stated that an appropriate range is from 1.0 to 100 in terms of n.moles EGF/n.mole iron present in the material. The appropriate ratio will of course depend on the efficiency of the material as an iron provider in vivo and ratios both below and above those quoted may be considered. By way of further guidance it may be stated that in the context of the present invention a daily dosage of EGF of 10 to 100 n.moles/kg of body weight is often appropriate, although once again doses outside this range may be considered. In addition to its use in pharmaceutical compositions the incorporation of EGF into foodstuffs may be considered, usually those already containing a source of iron. Such foodstuffs may take various forms, either liquid, semi-solid or solid, and may for example take the form of conventional human infant or piglet feed materials. Further examples of such piglet feed materials are to be found in the US Patent 4,362,710 referred to hereinbefore.

The present invention thus further includes a foodstuff which comprises epidermal growth factor and an iron-containing nutritional material.

Moreover, it will also be appreciated that the present invention includes a method for the treatment of a patient, particularly a human or other mammalian patient, which comprises administering to said patient epidermal growth factor and an iron-providing material in order to effect an increase in the level of iron in the patient's bloodstream.
The present invention further extends to the use of EGF in conjunction with other materials than iron—providing materials which are of value specifically in growth promotion or in maintaining general well being of a human or an animal, particularly a piglet. One group of such materials is those which provide one of the fifteen trace elements essential for human and animal well being. Many of these are transition elements and apart from iron other metallic trace elements of particular interest are chromium, and also manganese, cobalt, copper and molybdenum, as well as selenium and zinc. EGF may be used to enhance the uptake of the trace elements and effect an increase in the level of the element in the patient's bloodstream, particularly of cobalt, copper, selenium and zinc, in an essentially similar manner to that described herein for its use in the enhancement of the uptake of iron. Thus, pharmaceutical compositions and foodstuffs may be used containing EGF and a material providing one of these other trace elements or a plurality of materials providing different elements. Such materials may conveniently be chosen from those described in the art for providing the element in question and the pharmaceutical composition or foodstuff may conveniently, particularly in the case of a foodstuff, contain materials providing a range of these beneficial trace elements, including iron. Data on preferred human and veterinary dosage levels for the different elements is to be found in the literature but the use of daily dosages of EGF selected within the ranges quoted herein together with proportions of EGF and the element in question within the range quoted herein for iron will usually be broadly suitable.

In its second aspect the invention is illustrated by the following Example 4.
Example 1: Treatment of piglets with epidermal growth factor

Three-day old Large White piglets maintained on their mother's milk were injected subcutaneously every eight hours (08.00, 16.00, 24.00) for a period of 3 days with 20 μg/kg of synthetic mouse EGF (Wellcome) in 0.2 ml normal saline as a carrier (n=15) or with 0.2 ml/kg of the carrier (n=15). All of the animals were killed 8 hours after the last injection by stunning and exsanguination. The small intestine was removed and samples of the mid-intestine were taken for biochemical estimation of sucrase, maltase, lactase and alkaline phosphatase activities for the cytochemical determination of α-glucosidase activity and for the quantitation of crypt cell mitosis and total crypt cell population (Procedures as referred to in Smith, Annual Review of Physiology, 1985, 47, 247-260 and in The Biology of Epithelial Cell Population, Wright and Allison, Clarendon Press, Oxford, 1984) and as described in detail in Example 3(A).

Administration of this amount of EGF had no effect on the growth rate of the piglets (weight gain 0.22 ± 0.03 versus 0.20 ± 0.03 kg/day for control and EGF injected piglets respectively.) It should be appreciated that an increase in growth rate would not be expected at this stage since the solid feeding possible after the EGF treatment is completed had not been commenced.

The treatment was found to have caused a two-fold increase in sucrase and maltase activity without affecting the activities of lactase and alkaline phosphatase (estimated using p-nitrophenyl phosphate as a substrate), the values of the means ± SEM for each substrate being shown in the table together with the P values for the results with sucrose and maltose. These changes were accompanied by a significant (P < 0.005) increase in total but not mitotic crypt cell population (419 ± 13.1 versus 474 ± 9.6 cells and 11.2 ± 1.2 versus 12.50 ± 0.7 cells, respectively, for control versus EGF-injected piglets). Cytochemical analysis showed a 65% EGF-induced increase in α-glucosidase activity (sucrase plus maltase) in mid-villus enterocytes following treatment of the
3-day-old animals. An increase of 41% was observed in enterocytes 60 μm from the villus base of the piglets after the EGF treatment.

Table: Enzyme Activity

<table>
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<tr>
<th>Substrate</th>
<th>Control μ moles substrate/mg protein/hour</th>
<th>EGF injected piglets μ moles substrate/mg protein/hour</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>0.40 ± 0.05</td>
<td>0.83 ± 0.10</td>
<td>&lt;0.01</td>
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<tr>
<td>Maltose</td>
<td>0.56 ± 0.05</td>
<td>1.00 ± 0.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lactose</td>
<td>12.0 ± 1.5</td>
<td>11.0 ± 0.9</td>
<td>-</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>13.8 ± 2.7</td>
<td>13.1 ± 2.4</td>
<td>-</td>
</tr>
</tbody>
</table>

Example 2: Treatment of piglets with dexamethasone

A group of three, three-day old, Large White piglets maintained on their mother's milk were injected subcutaneously with 2 mg/kg of a slow release preparation of dexamethasone (Dexafort-Intervet), a further group of three piglets receiving 0.2 ml/kg of normal saline as a control. All of the animals were killed 8 hours after the end of the 3 day period by stunning and exsanguination. The small intestine was removed and samples of the mid-intestine were taken for determination of amino acid uptake (radioactively labelled alanine) in the presence of Na⁺ in cells located at measured distances along individual villi through microdensitometry of enhanced autoradiography (procedures referred to in Smith, Annual Review of Physiology, 1985, 47, 247-260) and as described in more detail in Example 3(B).
The results obtained are shown in the accompanying Figure which shows alanine concentration in mM as a function of distance of the point of measurement from the villus tip in μM for both the dexamethasone treated piglets and the control group. In both cases as expected the concentration is highest at the tip (distance 0 on the abscissa) and falls as the distance increases, but the particularly noteworthy result is that at all distances the alanine uptake is observed to be higher for the dexamethasone treated piglets, with a five-fold increase over the control being observed at the villus tip.

In a variation of the above experiment in which the piglets were treated with EGF as described in Example 1 no significant difference was seen in the level of alanine uptake for the EGF treated piglets as compared with the control.

Example 3: Comparison of the effects of epidermal growth factor, dexamethasone and combined epidermal growth factor/dexamethasone in the piglet

(A) Half litters of three-day-old Large White piglets maintained on their mother's milk were injected subcutaneously with EGF, dexamethasone or a mixture of EGF and dexamethasone, the other half of the litter being injected with equivalent volumes of carrier normal saline as a control. EGF was injected every 8 hours for three days in the form of 20 μg/kg of synthetic mouse EGF (Wellcome) in 0.2 ml of normal saline as carrier (60 μg/kg/day), while dexamethasone was given as a single injection on day three in the form of a slow release preparation (Dexafort-Intervet; 1.3 mg/kg dexamethasone phenyl propionate + 0.65 mg/kg dexamethasone sodium phosphate). All of the piglets were killed at six days of age by stunning and exsanguination. The small intestine was then quickly removed and samples taken 25, 50 and 75% along the length for biochemical determination of disaccharidase and alkaline phosphatase activities.
Lactase activities were estimated at three sites in the small intestine by incubating aliquots of intestinal homogenates at 37°C for 30 minutes with 90 mM lactose in 100 mM sodium citrate buffer, pH 6.0, containing 0.1 mM p-chloromercuribenzoate to inhibit lysosomal β-galactosidase (procedure as described by Koldovsky et al, Analytical Biochemistry, 1969, 27, 409-418). Sucrase and maltase activities were estimated by incubating intestinal homogenates with 90 mM sucrose or maltose at 37°C for 30-60 minutes in 90 mM NaCl, 4 mM disodium succinate buffer, pH 6.0. The homogenates were pre-heated at 55°C for 45 minutes before estimating maltase activity in order to destroy the ability of sucrase-isomaltase to hydrolyse maltose (procedure as described by Dahlqvist, Acta Chemica Scandinavica, 1960, 13, 1659-1667). Glucose released by the hydrolysis of substrates in these experiments was estimated using the glucose oxidase kit of Boehringer.

Alkaline phosphatase activity was determined by incubating aliquots of intestinal homogenates with 10 mM p-nitrophenyl phosphate at 37°C for 10 minutes in 50 mM Tris buffer, pH 10.1, containing 50 mM MgCl₂. Release of p-nitrophenol was measured in 0.5N NaOH by spectrometry (procedure as described by Hausamen et al, Clinical Chimica, 1967, 15, 241-245). All enzyme activities were finally related to the amount of protein present in homogenate determined according to the method of Markwell, Analytical Biochemistry, 1978, 87, 206-210.

The ability of EGF or dexamethasone to affect hydrolyase activities in homogenates of pig intestine was assessed, in multi-litter experiments, by analysis of variance. The ability of EGF and dexamethasone to produce similar effects when administered jointly to a single litter of piglets was assessed using an unpaired Student's t-test.

The results obtained are shown in Figure 2 in which the histograms labelled C correspond to the control, those labelled E to the EGF treatment, those labelled D to the dexamethasone treatment and those labelled DE to the combined EGF/dexamethasone
treatment. The notations 1*, 2*, 3*, 4* and 5* indicate a significant different between test and control piglets at the levels of \( p < 0.05 < 0.01 < 0.005 < 0.002 < 0.001 \), respectively. It will be seen that the sucrase and maltase activities are increased significantly in the mid small intestine by EGF treatment but not by dexamethasone treatment. EGF plus dexamethasone also causes a significant increase in sucrase and maltase activities in the distal small intestine, presumably because of the presence of EGF. The selectivity of the EGF stimulation of sucrase and maltase activities is emphasised by the lack of any significant change in alkaline phosphatase activities measured at any site in the small intestine.

(B) The procedure described in (A) was repeated but the control piglets were matched for weight with the piglets treated with one of EGF, dexamethasone and EGF/dexamethasone.

Pieces of piglet mid small intestine mounted mucosal face upwards in an apparatus to measure amino acid uptake were superfused initially with a sodium-free bicarbonate saline, gassed with 95% \( \text{O}_2/5\% \text{CO}_2 \), at a temperature of 37°C for a period of 5 minutes. This solution was then replaced either with an identical one containing 1 mM tritiated alanine (300 \( \mu\text{Ci/ml} \)) or by sodium-containing bicarbonate saline containing 1 mM tritiated alanine or lysine (100 \( \mu\text{Ci/ml} \) and 100-250 \( \mu\text{Ci/ml} \) respectively). All three solutions were then stirred vigorously at 37°C for 45 seconds before being replaced by a phosphate buffer containing glutaraldehyde to cross-link absorbed amino acids to cellular protein. Fixed tissue removed from the apparatus was processed for autoradiography, the density of silver grains in individual enterocytes then being determined in eosin stained sections using an M85 microdensitometer (Vickers Instruments). These measurements of optical density, carried out from villus tip to a position on the villus where readings became negligible, were later converted to estimates of intra-enterocyte amino acid concentrations by comparison against known gelatin standards.
This method of quantitation is very similar to that described by King et al., Journal of Physiology, 1983, 344, 465-481.

The results obtained are shown in Figure 3 in which E, D, DE, 1* and 2* have the meanings indicated for Figure 2 and L indicates the uptake of lysine whilst A+ and A- indicate the uptake of alanine in the presence and absence of sodium, respectively. It will be seen that EGF alone had no effect on lysine uptake or on the uptake of alanine measured in the presence or absence of sodium. Dexamethasone caused a two to threefold increase in alanine uptake measured in the presence of sodium but had no effect on alanine uptake measured in the absence of sodium or on lysine uptake. The ability of dexamethasone to cause a selective increase in sodium-dependent alanine transport was also observed following the administration of combined EGF/dexamethasone.

It will be seen from Figures 2 and 3, therefore, that a combination of EGF and dexamethasone is of value both in increasing maltose and sucrose activities and in increasing the uptake of alanine (measured in the presence of sodium).

Example 4: Enhancement of Uptake of Iron in Mice

(A) Six week old, male To mice received doses averaging 1 n.mole/day of mouse epidermal growth factor in their drinking water during a period of up to 7 days, a control group of similar mice receiving plain drinking water. Iron absorption was then studied in the mice by both in vitro and in vivo techniques. The former, using techniques as described by Raja et al., Cell Biochem. and Funct., 1987, 5, 69-76, involved incubation of intestinal fragments from the mice with a 0-450 μM solution of a Fe³⁺ chelate (the 2:1 nitritoltriacetic acid:iron(III) complex) whilst the latter, using techniques as described by Simpson and Peters, Biochim. Biophys. Acta., 1986, 856, 115-122, involved instillation of 50-100 μl of a 250 μM solution of the Fe³⁺ chelate into a tied-off loop of intestine of an anaesthetised mouse.
It was found that neither group showed a change in the wet weight of the duodenum per unit length but that cell turnover rates, as reflected by L-ornithine decarboxylase activity, were elevated in the duodenum of the animals which had received EGF. In vitro uptake studies also showed no change in the kinetic parameters for $^{59}\text{Fe}^{3+}$ uptake for EGF-treated animals $[K_m = 101 \pm 18(4); \ V_{\text{max}} = 9.4 \pm 1.1(4) \ \text{pmol/mg/min}]$ as compared with the controls $[K_m = 103 \pm 20(9); \ V_{\text{max}} = 10.5 \pm 0.9(9)]$. In vivo experiments showed a progressive increase in the total mucosal uptake of $^{59}\text{Fe}^{3+}$ in the EGF-treated animals which was maximal after 3 days of EGF administration. The data obtained is shown in the Table from which it will be seen that the enhanced uptake was due to increases in both the mucosal retention and carcass transfer.

In vivo studies were carried out with $^{51}\text{Cr}-\text{EDTA}$ (ethylene diamine tetra-acetic acid) using techniques as described by Bjamason et al., Gut, 1985, 26, 579-586 which involve instillation of 50-100 µl of a 100 µM $^{51}\text{Cr}-\text{EDTA}$ solution into a tied-off loop of intestine of an anaesthetised mouse. These showed an increased permeability in EGF-treated animals [total mucosal uptake $= 25.1 \pm 1.7(5) \ \text{pmol/ mg/10 min}]$ as compared with the controls [total mucosal uptake $= 9.6 \pm 1.1(5) \ \text{pmol/mg/10 min}; \ p < 0.001$]. These studies demonstrate that oral EGF feeding enhances intestinal proliferation and in vivo $^{59}\text{Fe}^{3+}$ absorption, although the latter is not via a specific carrier-mediated pathway.
TABLE (1)

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<th>Mucosal retention</th>
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<tr>
<td>Controls (20)</td>
<td>40.6 ± 2.3</td>
<td>22.9 ± 2.0</td>
<td>63.4 ± 3.4</td>
</tr>
<tr>
<td>EGF-treated</td>
<td>57.1 ± 6.3(2)</td>
<td>35.3 ± 2.6(2)</td>
<td>92.4 ± 8.4(3)</td>
</tr>
</tbody>
</table>

(1) Values are for mean ± SE pmol $^{59}$Fe$^{3+}$/mg tissue/10 minutes for number of animals indicated between parentheses.

(2) $p < 0.01$

(3) $p < 0.001$

(2) The procedure described under (A) was repeated but with the treated mice receiving the EGF for 3 rather than 7 days. After fasting for 12 hours both the EGF-treated and the control mice were treated intragastrically with 1 μCurie of $^{59}$Fe$^{3+}$ administered as 50 μl of a 100 μM aqueous solution of the radiolabelled 2:1 nitrilotriacetic acid:iron(III) complex. The mice were subjected to a whole body count by gamma counting at 3 hours and 7 days later. The percentage of the $^{59}$Fe$^{3+}$ present at 3 hours which was retained after 7 days was $12.9 ± 2.0$ for the controls and $21.0 ± 3.2$ for the EGF-treated mice thereby clearly indicating a markedly increased retention for the treated mice.
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CLAIMS

1. A composition comprising epidermal growth factor and an iron-providing material.

2. A method for the promotion of growth in the young of the pig and other farm animals which comprises administering to a young animal epidermal growth factor and dexamethasone or a physiologically active derivative of dexamethasone.

3. A method for the modification of the digestive system of the young of the pig and other farm animals which comprises administering epidermal growth factor to a young animal having an immature gut cell structure.

4. A method for the enhancement of nutrient uptake in an animal or human which comprises administering thereto dexamethasone or a physiologically active derivative thereof.

5. A method according to Claim 2, 3 or 4, in which the epidermal growth factor and/or dexamethasone or derivative thereof is administered to an animal which is a member of the group consisting of the pig, cattle, goat, sheep and horse.

6. A method according to Claim 5, in which the animal is a piglet.

7. A method according to any of Claims 2 to 6, in which the epidermal growth factor is mouse EGF.

8. A foodstuff comprising dexamethasone or a physiologically active derivative thereof together with a nutritional material.

9. A composition which comprises epidermal growth factor and dexamethasone or a physiologically acceptable derivative thereof together with a physiologically acceptable diluent or carrier.

10. A product comprising epidermal growth factor and dexamethasone or a physiologically active derivative thereof for simultaneous, separate or sequential use in growth promotion.

11. A kit for use in growth promotion comprising in association epidermal growth factor and dexamethasone or a physiologically active derivative thereof.

12. The use of epidermal growth factor for the manufacture of a medicament for use in effecting an increase in the level of iron in a patient's bloodstream.
13. The use according to Claim 12, in which the medicament additionally comprises an iron-providing material.
15. A product comprising epidermal growth factor and an iron-providing material for simultaneous, separate or sequential use in effecting an increase in the level of iron in a patient's bloodstream.
16. A kit for use in increasing the uptake of iron by the body which comprises in association epidermal growth factor and an iron-providing material.
17. A pharmaceutical composition comprising epidermal growth factor and an iron-providing material together with a physiologically acceptable diluent or carrier.
18. A composition according to Claim 1, 9 or 17, which is adapted for oral administration.
19. A composition according to Claim 1, 9 or 17, which is of an injectable form.
20. A foodstuff comprising epidermal growth factor and an iron-providing nutrient material.
21. A method for the treatment of a patient which comprises administering to said patient epidermal growth factor and an iron-providing material in order to effect an increase of the level of iron in the patient's bloodstream.
22. A method according to Claim 20, which is used for the treatment of the human or the piglet.
Fig. 1
Fig. 2
INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 87/00839

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) 4

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC4: A 61 K 37/36; A 61 K 31/57; A 23 K 1/18; A 23 K 1/165;
A 23 K 1/175; //(A 61 K 37/36, 33/26, 31:57)

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System | Classification Symbols
---|---
IPC4 | A 61 K; A 23 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 6

III. DOCUMENTS CONSIDERED TO BE RELEVANT 3

<table>
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<th>Category</th>
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<td>US, A, 3036917 (LESLIE D. HARROP) 29 May 1962 see column 1, lines 21-55; column 4, lines 20-27</td>
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<td>X</td>
<td>US, A, 3737535 (JOHN R. BRETHOUR) 5 June 1973 see column 2, lines 37-66; column 4, line 57 - column 6, line 9</td>
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<td>X</td>
<td>EP, A, 0134385 (SANWA KAGAKU KENKYUSHO CO., LTD) 20 March 1985 see page 10, line 11 - page 11, line 16</td>
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<td>A</td>
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<td>A</td>
<td>GB, A, 1123965 (A.B. EWOIS) 14 August 1968</td>
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* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 17th August 1988

Date of Mailing of this International Search Report - 5 SEP 1988

International Searching Authority EUROPEAN PATENT OFFICE

Signature of Authorized Officer P.C. VAN DER PUTTEN

Form PCT/ISA/210 (second sheet) (January 1985)
V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers XX, because they relate to subject matter not required to be searched by this Authority, namely:
   XX Claims 2-7, 21-22
   Please see PCT Rule 39.1(iv)
   Methods for treatment of human or animal body by means of surgery or therapy, as well as diagnostic methods.

2. Claim numbers .............., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claim numbers .............., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/R120 (supplemental sheet (2)) (January 1985)
ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. GB 8700839
SA 19576

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDOC file on 17/03/88. The European Patent Office is in no way liable for those particulars which are merely given for the purpose of information.

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For more details about this annex: see Official Journal of the European Patent Office, No. 12/82.