Cell kinetic studies in the epidermis of the mouse. I. Changes in labeling index with time after tritiated thymidine administration[1]

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Summary. The changes in the labeling index (LI) with time after a single injection of tritiated thymidine (^{3}HTdR) at each of 4 different times of the day have been studied. Slight differences occur in the shape of these LI curves, (e.g. in the timing of the peaks) depending on the time of day when the initial injection was given. Thus, the time of day influences not only the number of cells in DNA synthesis but also determines the subsequent behavior of the labeled cells. The curves show 3 distinct peaks from which estimates of the cell cycle time can be made. The technique permits the cell cycle time to be estimated. From the data as a whole a minimum cell cycle time of 90 h for basal cells in the epidermis on the back of a mouse is obtained. The technique also provides estimates for the duration of $S+G_2+M$ which varies depending on the time of day that the label is given. The LI curves can best be understood if the basal layer is assumed to contain 2 cell populations with differing cell cycle times; one having a long cell cycle (about 180 h) but short S-phase and containing the stem cells, the other having a short cell cycle (about 90 h) and a long S-phase duration and consisting of transit cells.

There have been, in the past, studies on the changes in labeling index (LI) with time after administration of tritiated thymidine (^{3}HTdR)[2-4]. These studies have usually involved relatively few sample times and/or a short overall time course and all made use of sectioned material. The results tended to illustrate the existence of an early first peak and a subsequent scatter of the data points which largely prevented any detailed analysis at the later times. However, studies of changes in the fraction of labeled cells in the basal layer should provide information on the kinetics of cell loss from the basal layer and the cell cycle time, as shown here. Using autoradiographs of sheets of epidermis large numbers of basal cells can be studied relatively easily and hence a detailed study was undertaken. As will be seen from the data presented here an estimate of the cell cycle time could be obtained. Detailed mathematical analysis[5] of this type of data together with results from other cell kinetic experiments provided information on cell loss, the age distribution and growth fraction, and permitted some conclusions to be drawn on the proliferative organization in the basal layer.

Materials and methods. Male DBA-2 mice between the ages 7 and 8 weeks were used throughout. Male animals of this age consistently have dorsal skin within which the hair follicles are dormant[6] and the epidermis is thus in a fairly stable proliferative state. The animals were housed 4 per cage in temperature, humidity and light (12 h light starting at 06.00 h) controlled rooms with a continuous supply of food and water. For some experiments animals were placed in a room with the light cycle reversed (i.e. the lights were switched off at 06.00 h). In all cases the animals were left undisturbed in their respective rooms for a period of 4 weeks prior to the beginning of an experiment, so that they were thoroughly acclimatized. Previous labeling studies had confirmed that the reversal of the light cycle reversed the labeling index circadian pattern.

Methyl-labeled tritiated thymidine at a sp. act. of 5 Ci/mM (Amersham International Inc.) was injected i.p. at a dose of 25 µCi per mouse. Since each mouse weighs about 20 g this corresponds to about 1.25 µCi/g. The ^{3}HTdR was given to groups of mice at either 03.00, 09.00, 15.00 or 21.00 h, these times subsequently being used to classify the experiments. Groups of at least 4 mice were sampled at various times after ^{3}HTdR administration. Several parts of the experiment were repeated over a period of 3 years, the results eventually all being pooled for the final presentation of the data. Hence, for some time points as many as 24 mice were used i.e. 6 repeats of a given point.

On killing, the hair was clipped from the back of the mouse and a large area of skin was fixed to a sheet of paper and the hair stubble removed using a commercial depilatory (Immac, Anne French, London). The piece of skin was then carefully washed, rolled up with the attached paper and placed in 0.5% acetic acid at 4 °C for about 24 h. The acetic acid was then replaced with chilled Carnoy's fixative for 20–30 min and finally stored in 70% ethanol until needed.

Sheets of pure epidermis were removed using fine forceps and a dissecting microscope. These sheets were placed keratin layers downward on gelatin coated microscope slides and air dried. They were then Feulgen stained prior to dipping in K5 emulsion (Ilford Ltd, Basildon) diluted

Table 1. Changes in the labeling index over the 1st 2 h

Treatment (^{3}HTdR dose)	Time of day of ^{3}HTdR injection	Time of sampling after ^{3}HTdR	LI% ± SE		Number of mice	
25 µCi	03.00	40 min	6.5 ± 0.4(100)	5.8 ± 0.4(100)	23	4
		1.5 h	3.8 ± 0.3(58)		4	
		2 h	5.0 ± 0.2(77)	4.6 ± 0.3(79)	10	4
5 µCi		40 min	5.1 ± 0.8(100)		4	
		2 h	4.7 ± 0.3(92)		4	
100 µCi		40 min	5.4 ± 0.4(100)		4	
		2 h	4.7 ± 0.4(87)		4	
25 µCi	09.00	40 min	3.3 ± 0.4(100)		4	
		2 h	5.0 ± 0.5(152)		3	
25 µCi	15.00	40 min	3.2 ± 0.2(100)		23	
		2 h	2.5 ± 0.3(78)		6	
25 µCi	21.00	40 min	3.3 ± 0.4(100)		8	
		2 h	3.9 ± 0.4(118)		4	

Figures in brackets show the percentage increase or decrease when the 2 h sample is compared with the 40-min sample.

1:1 with distilled water. The dipped slides were air dried and exposed, usually for 4 weeks, at 4 °C. After developing the slides were dehydrated and mounted. Three random fields were selected and the number of labeled (3 grains or more) and unlabeled basal cells counted using an eyepiece (Whipple)grid. The area counted was 0.03 mm^2 hence a minimum area of 0.09 mm^2 was scored per mouse, or about 1000 basal cells since there are about 10^4 basal cells/mm^2. The labeling index was determined for each mouse and the mean for each group determined with its SE. The data were plotted and lines were fitted by eye.

Results. The results are presented in figures 1–4 where the changes in labeling index are plotted against time. The time of day when the initial injection of tritiated thymidine was given varied in the 4 experiments from 03.00 h (fig. 1) to 09.00 h (fig. 2), 15.00 h (fig. 3) and 21.00 h (fig. 4). The following points can be made about the data shown in figures 1–4.

1. 2 h after an initial injection of ^{3}HTdR at 03.00 or 15.00 h there is a lower LI (by about 22%) than immediately (40 min) after the injection (see also table 1). The LI may be even lower 1.5 h after the injection. In contrast the 09.00 and 21.00 h experiments show higher LI-values at 2 h than at 40 min (table 1).
2. There is then a progressive rise in LI until values double the initial (40 min) LI are reached by 9–20 h (table 2). There is a broad peak in LI that starts at about the same time but may contain some structure with a minor trough at 18–30 h. The LI-values during the broad 1 st peak often exceed values that are twice the initial LI (e.g. see figs 1 and 2).
3. The LI gradually decreases with time permitting a 2nd broad peak to be detected at 80–140 h (see table 2) in all but possibly the 21.00 h experiment.
4. There is a further fall in LI with a less well defined 3rd peak at 190–220 h.
5. The LI continues to fall reaching LI-values of about 1% at times in excess of 300 h with no further evidence of any peaks.
6. The effect of increasing the autoradiographic exposure time is minimal for early times (fig. 1) and often slightly variable. A doubling of exposure time at later times results in a slight increase in the levels of the LI but does not change the general trends.

Discussion. A similar type of experiment was conducted by Hegazy and Fowler[3] using sectioned material, fewer time-points and a shorter overall time scale. However, few comparisons can be made with the present experiments. An early fall in the LI was observed between 4 and 8 h, at a time when the LI was rising in all the present experiments. The 1st peak occured somewhat earlier in Hegazy and Fowler's experiment at about 9 h. Gibbs and Casarett[2]

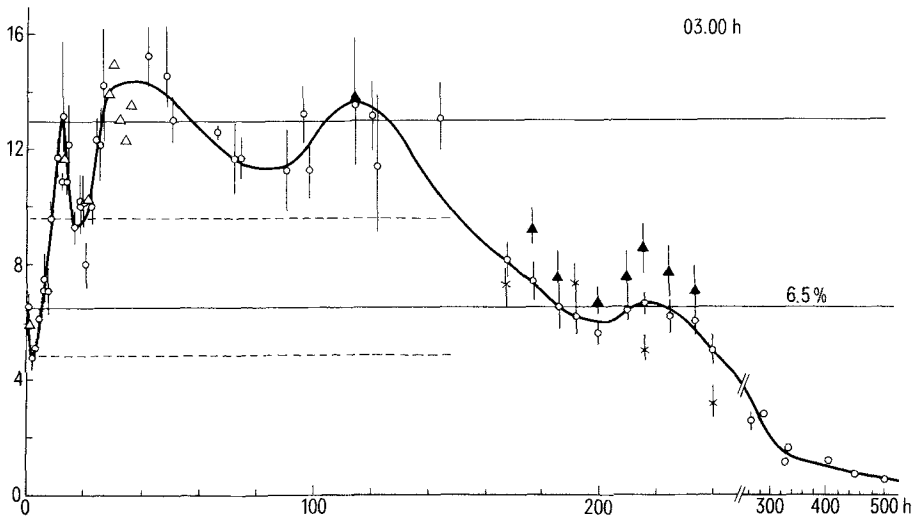

Figure 1. Changes in labeling index with increasing time after an injection of 25 µCi ^{3}HTdR at 03.00 h. Each point represents the mean and SE of at least 4 mice (in some cases as many as 23 mice were used). Altogether 285 were used for the main experiment with a further 72 in experiments using longer autoradiographic exposure times. The results of 2 separate experiments conducted at different times are shown by the open circles and triangles. The crosses represent results using 6 week instead of the usual 4 week exposures while the closed triangles represent results using 8 week exposures. The horizontal solid lines are drawn at values equalling the initial LI and double the initial LI while the dashed lines are drawn at the minimum LI and double the minimum LI.

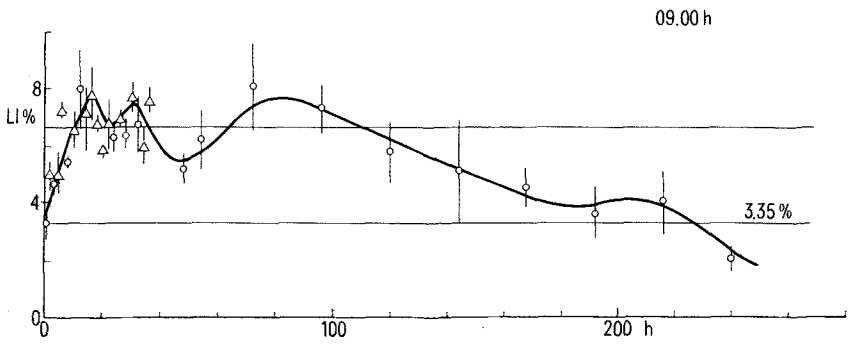

Figure 2. Changes in labeling index with increasing time after an injection of 25 µCi ^{3}HTdR at 09.00 h. Each point represents the mean of between 4 and 8 mice while the whole graph represents the results from 118 mice. Symbols and further description as for figure 1.

conducted a similar experiment on the epithelium of hamster cheek pouch. This also showed an initial drop in LI and an early 1st peak with some evidence of sub-structure followed by a progressive decline in LI with time.

The early fall in labeling index. Similar observations to those presented here can be seen in the literature (e.g. Gibbs and Casarett[2] reported a 40% drop in LI 2 h after labeling while Olsson[4] reported a 60% drop). In our experience this early fall is somewhat variable in size and reproducibility (table 1). This early fall in LI is hard to explain at present. Some cells may temporarily move slightly suprabasally and hence; if labeled, be lost from epidermal sheets. However, this seems an unlikely explanation since a similar drop is observed in sectioned material. Alternatively cells may be killed by internal β-irradiation. However, the number of cells killed by similar tracer doses of ^{3}HTdR in the intestine can be counted and appears to be a very small percentage of all the S-phase cells[7,8]. However,

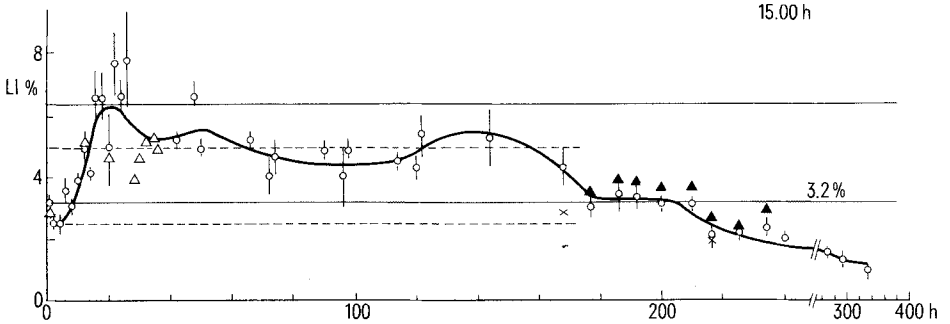

Figure 3. Changes in labeling index with increasing time after an injection of 25 µCi ^{3}HTdR at 15.00 h. Each point represents the mean of between 4 and 23 mice while the whole graph represents the results from 219 mice. A further 50 mice were used in an experiment to test different exposure times. Symbols and further description as for figure 1.

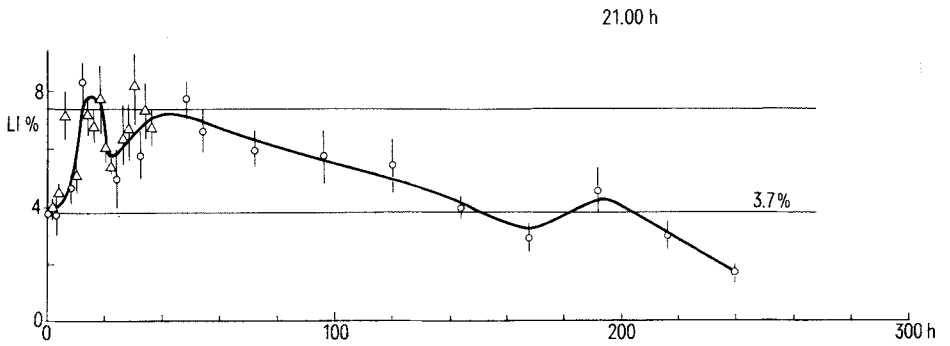

Figure 4. Changes in labeling index with increasing time after an injection of 25 µCi ^{3}HTdR at 21.00 h. Each point represents the mean of between 4 and 8 mice while the whole graph represents the results from 126 mice. Symbols and further description as for figure 1.

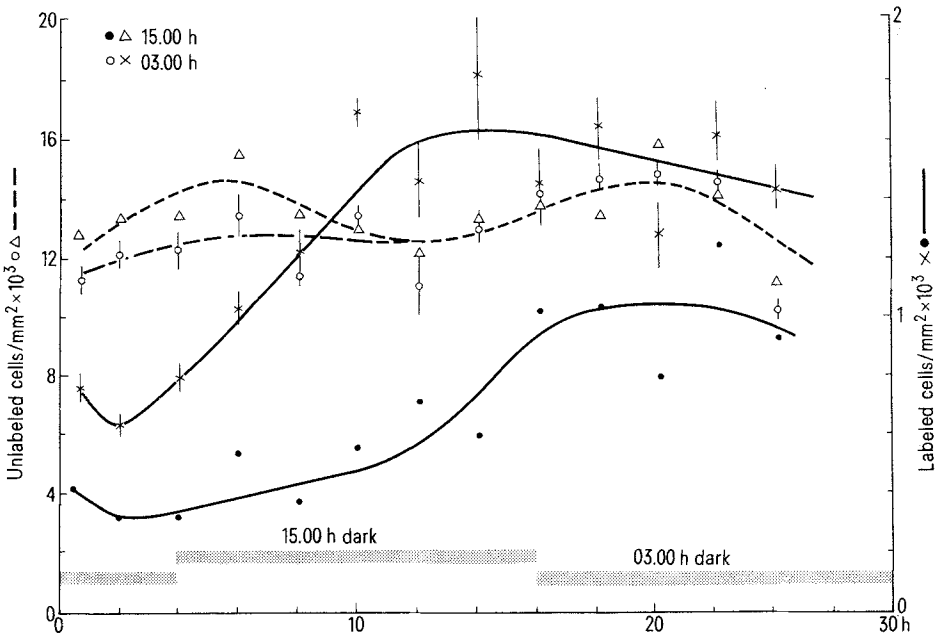

Figure 5. Changes in the absolute number of unlabeled (open circles and triangles) and labeled (crosses and closed circles) cells per mm^2 of epidermis with time after an injection of 25 µCi of ^{3}HTdR given at either 03.00 or 15.00 h. Typical SE are shown by the 03.00 h data (open circles and crosses), but are omitted from the 15.00 h data (closed circles and triangles). The periods of darkness are shown for the 2 experiments, 15.00 h (upper), and 03.00 h (lower).

such cells do die rapidly so the time scale for cell death and the drop in LI are compatible. The fall in the LI does not appear to be influenced by the size of the ^{3}HTdR dose which is surprising if the fall is due to radiation induced cell death. If the drop is due to cell death, the subsequent rise in LI should not exceed a value twice the minimum value. In fact it usually exceeds even twice the initial LI. This can only be explained if the killed cells release labeled DNA catabolites that are rapidly and efficiently reutilized. (Some of these points have been discussed elsewhere in particular with reference to circadian rhythms[9]. It is hard to understand why cells labeled at 09.00 or 21.00 h are not killed if those labeled at 03.00 or 15.00 h are killed (see table 1)). The only other possible explanation would be that radioactivity is somehow lost by some nuclei at 2 h after first becoming insoluble at 40 min. Perhaps for some cells the membranes become permeable to some post-phosphorylation metabolites. Since this loss of label appears to be influenced by the circadian rhythm, the membrane permeability or the stability of some enzymes would have to be influenced by the time of day. These explanations are all made more difficult by the fact that the loss of label occurs at both the time of the peak and the trough in overall labeling. Figure 5 shows that the fall in labeling index cannot be completely explained by circadian fluctuations in the absolute number of unlabeled cells. These may vary slightly with time but the absolute number of labeled cells also shows a fall at 2 h (particularly for the 03.00 h data).

The 1st peak in LI. If it is assumed that the initial LI provides a reliable estimate of the fraction of cells in the S-phase and that none of these cells dies quickly, then the time taken to reach an LI-value double the initial LI is a measure of the total duration of S, G_2 and M. This appears to vary amongst the 4 experiments with those cells labeled at 09.00 h having the shortest value and those labeled at 15.00 h the longest (table 2).

It is not clear why the LI falls to provide a minor trough at 18–30 h. There is no evidence in the literature to support the idea that this may be due to a few cells with a very short cell cycle (e.g. 20–24 h). This trough may be present in all the published data, though it is hard to be sure in some cases[2–4]. It is possible that circadian variations in the numbers of unlabeled cells as a consequence of circadian rhythms in either cell division or cell migration may account in part for the trough. The results shown in figure 5 represent only one experiment within the cumulative data shown in figures 1 and 3.

The 03.00 and 09.00 h data show LI-values 11.5% above a value twice the initial LI (figures 1 and 2). The other 2 experiments may show similar, though less pronounced, trends. The reason for this is again obscure but may be due in part to the circadian rhythms outlined above but is more likely to be the consequence of the delayed uptake of ^{3}HTdR into a few basal cells from an intracellular pre-replicative long-lived pool[10–12].

Hence, some labeled cells may be killed by the internal radiation and release reutilizable material, some may fail to divide, some may be rapidly lost after division to the suprabasal layers, while yet others may undergo delayed uptake over a relatively prolonged period. These processes may all contribute to a broadening of the 1st peak and in some cases to labeling indices that exceed double the initial LI.

The 2nd and 3rd peaks and cell cycle times. In all experiments the LI falls with increasing time and eventually reaches values equal to the initial LI at about 196 h (180–230 h). However, before reaching this level intermediate rises can be detected between 82 and 144 h in 3 of the experiments (figs 1–3). The appearance of this 2nd peak can be assumed to represent the 2nd division of at least some of the initially labeled cells and hence the timing of the 2nd peak can be used to estimate the cell cycle time of at least some of the basal cells, which as can be seen from table 2 is about 85 h.

With increasing time the LI continues to fall but in at least 3 out of the 4 experiments some sign of a 3rd peak can be seen at about 190–220 h. The 3rd peak can be assumed to represent the 3rd division of some of the initially labeled cells (or possibly a 2nd division of some of the initially labeled cells: see below). The interval between the 1st and 3rd, and between the 2nd and 3rd peaks can also be used to provide further information on the cell cycle time (table 2).

Taking all the data together, the minimum cell cycle time for epidermal basal cells can be deduced using this technique to be about 90 h which is in agreement with various other estimates using other techniques[2–4, 13–15].

If the basal layer is assumed to contain a homogeneous stem cell compartment with a random loss of labeled cells through the cycle (possibly with a few non-dividing differentiating cells), then the LI should have continued to give a series of peaks reaching about the same value (dependent on the circadian rhythm), with the LI dropping back to the initial value between peaks until the radioactivity is reduced to sub-threshold levels. This reduction can be compensated for by prolongation of the autoradiographic exposure time. Since the LI did not remain at high levels even after extended exposure times (see figs 1–4), this would seem to be an unacceptable model for the proliferative organization of the basal layer.

Some of the data presented here have recently been mathematically modelled together with percent labeled mitosis and continuous labeling studies[5]. The conclusions from this modelling were that the basal layer contained 2 distinct subpopulations characterized by differing cell cycle times. Furthermore, one of the subpopulations contained the stem cell compartment and this subpopulation had a cell cycle

Table 2. Time of peaks in labeling index

Time of day of ^{3}HTdR injection	Time taken to double initial LI (h)[a]	Time of 1st peak (P_1) (h)[c]	Time of 2nd peak (P_2) (h)	P_2-P_1[d]	Time of 3rd peak (P_3) (h)	P_3-P_2[e]	P_3-P_1[f]
09.00	9	23(16–30)	82	59	210	128	187
15.00	20(10)[b]	36(20–52)	144	108	204	60	168
21.00	14	28(14–42)	–	–	192	–	164
03.00	12(7)[b]	25(12–38)	114	89	218	104	193
Mean values	13.75	28	113	85	206	97	178

[a] Probably equivalent to $T_S + T_{G_2} + T_m$; [b] time taken to double minimum LI at 2 h; [c] 1st peak may be structured. The value shown is midway between the minor peaks shown in brackets; [d] assumed to represent the cell cycle time of the 1st cell cycle; [e] assumed to represent the cell cycle time of the 2nd cell cycle; [f] assumed to represent the sum of the 1st and 2nd cell cycle times, or the cell cycle time of the 1st cell cycle for some cells (see text). Average cell cycle times (85 + 97)/2 = 91 h, 178/2 = 89 h if the basal layer is homogeneous or (91 + 178)/2 = 134 h if it is heterogeneous (see text).

time twice that of the 2nd transit cell compartment. Thus, the 3rd peak would be expected to contain cells that in fact were undergoing their 2nd division (in which case their cell cycle time would be about 180 h) as well as some cells undergoing their 3rd division.

The model which best fitted all the data was one with about 12% of the basal cells being stem cells (with a cell cycle time of 180 h). The remainder of the basal layer consisted of transit proliferative cells and cells awaiting migration (post-mitotic?). The model, together with other considerations, suggested that there might be 3 transit divisions with the cell cycle time in the 1st transit population similar to that of the stem cell (i.e. 180 h) while for the following 2 transit divisions the cell cycle time would be about 90 h. The average basal layer residence time of the post-mitotic cells was concluded to be 45 h with a half-life of 30 h[16,17].

By about the 3rd, or 4th day after labeling some labeled cells in the granular layer would begin nuclear degradation which could release radioactive precursors that might be reutilized. This might result in the appearance of some new weakly labeled cells. This process has not been considered in the discussion above. The presence of clear labeled mitoses at the time of the peaks in LI, and the occurrence of labeled pairs, triplets, quads etc. at times that correspond to the peaks in LI suggests that reutilization alone is not likely to account for the appearance of the peaks in labeling.

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Infusion of a novel peptide, PHI, in man. Pharmacokinetics and effect on gastric secretion

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Summary. PHI, infused in man, achieved plateau plasma levels of 297 pmoles/l. The plasma half life was 3.1 min, metabolic clearance rate was 16.4 ml/kg/min and estimated volume of distribution was 73.2 ml/kg. No subjective side effects were noted during the infusion and there was no significant alteration in submaximal pentagastrin stimulated gastric acid or pepsin secretion.

Tatemoto and Mutt have recently isolated a 27 amino-acid peptide from porcine intestine[3,4]. This peptide has been given the abbreviated name PHI, referring to the peptide (P) having N terminal histidine (H) and C terminal isoleucine amide (I). PHI has sequence homologies with members of the glucagon-secretin family and has thus been included in this family of peptides[3,4].

There is only limited information regarding the biological activity of PHI and its physiological role in man has not yet been defined. Its in vitro actions so far are also shared by other members of the glucagon-secretin family, for example stimulation of pancreatic exocrine secretion[5], activation of isolated acinar cells[6] and release of insulin and glucagon from isolated rat pancreas[7]. Recently PHI has been shown to have powerful effects on the stimulation of intestinal secretion and thus mimics actions of vasoactive intestinal peptide (VIP)[8,9].

This study was designed to investigate the pharmacokinetics of infused natural PHI in man, and determine its effect on gastrointestinal hormones in vivo, and monitor its effects on gastric secretion of pepsin and acid.

Methods. Highly purified PHI was isolated at the Karolinska Institute. It was prepared immediately before use, being dissolved in 0.9% sterile saline containing 1% human serum albumin.

PHI was measured by a recently developed radioimmunoassay. Briefly, the antibody was raised to porcine PHI coupled to bovine serum albumin (BSA) with glutaraldehyde, and was used at a final dilution of 1/10,000. The iodinated (^{125}I) PHI was prepared using the Iodogen method. The assay did not significantly crossreact with other members of the glucagon-secretin family, and could detect changes of 10 pmoles/l plasma with 95% confidence[10].

Plasma taken before and during the infusion of PHI was also assessed for concentration of pancreatic polypeptide, motilin, insulin and pancreatic glucagon[11].

Five healthy male subjects (aged 24–31) were studied. Full