The gastro-ileal digestion of $^{15}$N-labelled pea nitrogen in adult humans

BY NICOLAS GAUSSERÈS, SYLVAIN MAHÉ*, ROBERT BENAMOUZI, CATHERINE LUENGO, HENRIETTE DROUET, JACQUES RAUTUREAU AND DANIEL TOMÉ

1 Institut National de la Recherche Agronomique, Unité de Nutrition Humaine et de Physiologie Intestinale, Faculté des Sciences Pharmaceutiques et Biologies, 4 avenue de l’Observatoire, 75006 Paris, France
2 Service de Gastro-entérologie, Hôpital Avicenne, 125 route de Stalingrad, 93000 Bobigny, France

(Received 25 September 1995 - Revised 14 November 1995 - Accepted 30 November 1995)

The aim of the present study was to determine the gastro-ileal behaviour of pea protein in humans. For this purpose, twelve healthy volunteers were intubated with an intestinal tube located either in the jejunum ($n=5$) or the ileum ($n=7$). After fasting overnight, they ingested $195 \text{ mmol N of } [^{15}\text{N}]\text{pea.}$ Intestinal samples were collected for $6 \text{ h}$ in the jejunum and for $8 \text{ h}$ in the ileum. Before meal ingestion the basal liquid flow rate (ml/min) was $2.01 (SD 0.31)$ in the jejunum and $2.02 (SD 0.33)$ in the ileum. After meal ingestion the liquid phase of the meal peaked in the $40-60 \text{ min}$ period in the jejunum and in the $150-180 \text{ min}$ period in the ileum. The jejuno-ileal transit time of the liquid phase of the meal was $102 \text{ min}$. The basal flow rate of endogenous N (mmol N/min) was $0.22 (SD 0.15)$ in the jejunum and $0.16 (SD 0.10)$ in the ileum. The endogenous N flow rate peaked significantly ($P < 0.05$) in the jejunum in the $40-60 \text{ min}$ period whereas no stimulation of endogenous N could be detected in the ileum after meal ingestion. A significantly increased ($P < 0.05$) concentration of exogenous N was detected in the jejunum during the $20-30 \text{ min}$ period and during the $90-480 \text{ min}$ period in the ileum. The overall true gastro-ileal absorption of pea N was $89.4 (SD 1.1)$% with $69 (SD 14)$% absorbed between the stomach and the proximal jejunum and $20.4$% between the proximal jejunum and the terminal ileum. The percentage of ethanol-insoluble fraction (PN) in the exogenous N at the terminal ileum increased significantly ($P < 0.05$) to $75$% after $360 \text{ min}$. These results suggest that heat-treated pea protein has a digestibility close to that of animal protein.

Stable isotope: Vegetable protein: Intestinal absorption

The bioavailability of dietary proteins, i.e. the proportion of amino acids available for metabolic utilization, is mainly determined by digestion and absorption processes in the gastrointestinal tract. Both the nature of the protein and its state of processing before ingestion, as well as the nature of the other components of the meal, affect the different steps of protein assimilation, including gastric digestion and emptying, small-intestinal digestion, absorption, intestinal transit and colonic metabolism (Alpers, 1987; Young & Pellet, 1994). In addition, a large amount of endogenous protein is secreted into the intestinal lumen and is mixed with dietary N during the course of digestion (Gaudichon et al. 1994). The magnitude of this secretion is influenced by the nature of the meal, including the DM, energy and protein contents and the type and level of dietary fibre (Lurie et al. 1973; Girard-Globa et al. 1980; Vahouny, 1987; Corring et al. 1989).

Initially the proteins are partially degraded at the gastric level and progressively released

* For reprints.
in the duodenum (Alpers, 1987). The gastric emptying stage has been demonstrated previously to be the main step that regulates the kinetics of N absorption from highly digestible proteins such as milk protein (Gaudichon et al. 1994). Next, during their transit from the duodenum to the terminal ileum, both dietary (exogenous) and secreted (endogenous) proteins are hydrolysed by pancreatic and brush-border membrane enzymes and progressively absorbed. The kinetics of this absorption are probably an important factor in the control of amino acid delivery to the tissues for body protein synthesis. Finally, a fraction of the protein, which enters the large intestine, is either degraded with the production of NH₃ (which is subsequently absorbed and excreted as urinary N) or incorporated into microbial proteins and mainly excreted in the faeces (Zebrowska et al. 1978). It is considered that this fraction is of limited nutritional value to the host (Rowan et al. 1994).

In general, legume proteins, including pea protein, in their natural forms are considered to be less digestible than animal proteins. This reduced digestibility is difficult to explain as it results from both the structure and composition of the protein as well as the presence of fibre associated with the protein (Vahouny, 1987; Begbie & Putzai, 1989). Moreover, vegetable proteins are often associated with antinutritional factors which could both impair protein digestibility and bioavailability and increase losses of endogenous N (Grant, 1989). Thermal processing both improves the protein quality and the digestibility of vegetable proteins and reduces antinutritional activities (Young & Pellet, 1994). However, little information is available on the precise digestive patterns of these proteins in humans. The traditional faecal method used to determine dietary amino acid digestibility in humans does not take into account the complex series of metabolic and physiological transactions that take place in the gastrointestinal tract during the course of meal protein digestion, including intestinal digestion kinetics, amino acid absorption, endogenous N secretion levels, and the sizes of the protein and amino acid fractions entering the large intestine.

The aim of the present study was to analyse precisely the digestive pattern and the course of digestion and absorption of pea protein in the human intestine. For this purpose, heat-treated 15N-labelled pea flour was given orally to healthy volunteers equipped with either a jejunal or an ileal tube. Digesta samples were collected after meal ingestion and further analysed to differentiate between endogenous (unlabelled) and exogenous (15N-labelled) N. This approach allows determination of the true digestibility and absorption of the dietary protein at the different intestinal levels, i.e. proximal and distal small intestine.

MATERIALS AND METHODS

Diets

Pea (Pisum sativum) seeds of Solara cultivar were grown under controlled conditions, using 15NH₄Cl as fertilizer, and were kindly supplied by Professor A. Thevis (Gembloux, Belgium). The 15N-labelled whole peas were ground into flour, mixed with pure water (flour–water 1:6, w/v), cooked at 100°C for 1 h and then lyophilized. Each meal contained 75 g pea flour (195 mmol N), 300 ml water and 15 g polyethylene glycol 4000 (PEG-4000) used as a non-absorbable marker of the meal’s liquid phase.

Subjects

The studies were performed on twelve healthy volunteers. They were selected according to the following criteria: (1) no history of gastrointestinal surgery; (2) absence of gastrointestinal system disorders; (3) absence of pregnancy and (4) a stable, satisfactory nutritional status and a stable body weight. The anthropometric details of the twelve healthy adult subjects studied are summarized in Table 1. The study was composed of two
Table 1. Subjects' characteristics

<table>
<thead>
<tr>
<th></th>
<th>Males (n=8)</th>
<th>Females (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>21-39</td>
<td>28</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.70-1.86</td>
<td>1.80</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>64-90</td>
<td>73</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.5-26.3</td>
<td>22.7</td>
</tr>
</tbody>
</table>

groups: (a) digesta collected in the jejunum of five volunteers (four males and one female) ranging in age from 21 to 31 years (26 (SD 3) years) and in weight from 61 to 90 kg (73 (SD 10) kg); (b) digesta collected in the ileum of seven healthy volunteers (four males and three females) ranging from 19 to 39 years of age (28 (SD 7) years) and weighing from 46 to 77 kg (64 (SD 10) kg). The protocol was previously approved by the Ethical Committee of the St Germain-en-Laye Hospital (78100 St Germain-en-Laye, France). All subjects gave their consent for their participation in the study.

Intestinal perfusion technique and experimental design

An intestinal tube was passed from the nose to the small intestine of the volunteers as previously described (Mahé et al. 1992). The intestinal tube was used (1) to perfuse phenol red (PSP), a non-absorbable marker, into the intestine to calculate the flow rate and (2) to aspirate intestinal contents, 200 mm distally from the perfusion site. The perfusion site of PSP was located either at the Treitz angle or in the ileum. On the day before the test, subjects, after fasting, arrived at the hospital and the intestinal tube was positioned under radioscopic control. They had dinner at 20.00 hours and then fasted overnight. On the morning of the study the position of the intestinal tube at the beginning of the jejunum (i.e. 1.20 (SD 0.19) m from the nose) or at the distal ileum (i.e. 1.90 (SD 0.19) m from the nose) was checked under radioscopic control. Subjects were given the test meal (195 mmol N), each subject serving as his or her own control, and the sampling period lasted for either 6 h (jejunum) or 8 h (ileum). Starting before meal ingestion and continuing throughout the test period, a saline solution (130 mM-NaCl, 30 mM-mannitol, 5 mM-KCl) containing PSP (400 mg/l) was perfused into the intestine at the rate of 1 ml/min to calculate the intestinal flow rate. Intestinal samples were obtained by continuous suction through the distal opening of the intestinal tube. Aspirates were collected over ice and pooled at intervals of 20 min (jejunum) or 30 min (ileum). The 20 or 30 min before meal ingestion were considered to be the initial period (basal). Subjects were not allowed to ingest food or fluids during the remainder of the collection period.

Sample treatment

The volume and pH of digesta samples were measured after homogenization. The effluents were treated by the protease inhibitor 0.1 mM-diisopropylfluorophosphate (Sigma, Saint-Quentin-Fallavier, France), then frozen at −20°C and freeze-dried. For the separation of protein and non-protein N, ethanol (700 ml/l) and hexane were added to the portions of the DM of the effluents as previously described (Mahé et al. 1994b). The protein was pelleted by centrifugation at 2400 g for 25 min at 4°C. The pellet was believed to be made up of proteins (protein N, PN) and the supernatant fraction to contain peptides and free amino acids (non-protein N, NPN).
Sample analysis

The PEG-4000 and the PSP were measured by a turbidimetric method (Hyden, 1955) and by a spectrophotometric method (Scheld, 1966) respectively. The total N content was determined using a N analyser (NA 1500 series 2, Fisons Instruments, Manchester) with atropine being the standard. The $^{15}$N:$^{14}$N isotope ratio was determined by isotope-ratio mass spectrometry. A portion of each freeze-dried sample (i.e. jejunal or ileal effluent) was burned in the presence of purified O$_2$ in the combustion unit of an elemental analyser (NA 1500 series 2, Fisons Instruments) at 1020°C. The NO$_x$ from the burning process was reduced over Cu to N$_2$ at 650°C. The combustion unit was coupled with an isotope-ratio mass spectrometer (Optima, Fisons Instruments). The isotope ratio of N$_2$ was measured with reference to a calibrated $^{15}$N:$^{14}$N N tank.

Calculations and statistical analysis

Data points, representing the mean of each 20 min (jejunum) or 30 min (ileum) sampling period, were plotted after each period. The intestinal flow-rate was calculated from the PSP concentration in both the effluents and the perfusion solution and then was corrected for the perfusion flow rate (1 ml/min). The exogenous N (N$_{exo}$) in the digesta was calculated from the total N (N$_{tot}$) and the isotopic ratio $^{15}$N:$^{14}$N determined in both the digesta (E$_{dig}$) and the $[^{15}$N]pea (E$_{pea}$) according to the following formula: N$_{exo}$ = N$_{tot}$ x E$_{dig}$/E$_{pea}$. The endogenous N in the digesta (N$_{end}$) was calculated from the difference N$_{end}$ = N$_{tot}$ - N$_{exo}$.

A model curve calculated from the experimental cumulative quantity of exogenous N which passes both the proximal jejunum and the terminal ileum was obtained in the postprandial period (SigmaPlot 5.00, Jandel Corporation). Under these conditions the curve is in the form: $y = b/[1 + \exp(c(t-a))] + d$, in which $t$ is time, and $a$, $b$, $c$ and $d$ are parameters calculated from the model: $a$ represents the inflexion point, $b + d$ represents the value of the plateau and $c$ is related to the intestinal transit of the meal (the higher the absolute value of $c$, the more rapidly the plateau is reached). Results are expressed as means and standard deviations. To estimate differences between basal values and absorptive values within a period, statistical analysis was performed using Tukey's studentized range test (SAS 6.03, Statistical Analysis Systems Institute, Inc., Cary, NC, USA) and a probability of $P < 0.05$ was considered to be significant.

RESULTS

Flow rate of intestinal effluents and transit of the liquid phase of the meal

The intestinal effluent flow rate was measured both in the jejunum and in the ileum after pea ingestion (Fig. 1). Before meal ingestion the basal flow rates were 2.01 (SD 0.31) ml/min ($n$ 5) and 2.02 (SD 0.33) ml/min ($n$ 7) in the jejunum and in the ileum respectively. After meal ingestion the jejunal flow-rate increased significantly ($P < 0.05$) in the 40–100 min period, peaked to 9.94 (SD 2.99) ml/min in the 40–60 min period and then returned to a basal rate of 2.15 (SD 0.90) ml/min after 320 min. After meal ingestion the ileal flow-rate increased significantly ($P < 0.05$) in the 90–180 min period, peaked to 3.04 (SD 0.64) ml/min in the 150–180 min period and then progressively returned to a basal level of 1.16 (SD 0.80) ml/min after 390 min. During the 360 min after pea ingestion the volumes of the effluents recovered in the jejunum and in the ileum were 1186 (SD 305) and 755 (SD 188) ml respectively. After 480 min the volume of the effluents recovered in the ileum was 848 (SD 201) ml.

The transit time of the liquid phase of the meal was calculated from the recovery of the non-absorbable marker PEG-4000 at the collection site (i.e. jejunum or ileum) (Fig. 2). After meal ingestion the liquid phase of the meal increased significantly ($P < 0.05$) in the
Fig. 1. Effluent flow-rate profiles in (a) human jejunum and (b) ileum after the ingestion of 75 g pea flour/300 ml. Each value represents the mean of five subjects for the jejunum and seven subjects for the ileum; standard deviations are represented by vertical bars. The arrows represent the point of pea ingestion. * Mean values were significantly different from the basal value (t test, $P < 0.05$).

40–220 min period in the jejunum and in the 90–390 min period in the ileum. The time for 50% delivery of PEG was 89 min in the jejunum and 191 min in the ileum, thus giving a jejuno-ileal transit time for the liquid phase of the meal of 102 min.

**Endogenous and exogenous nitrogen in intestinal effluents**

N was measured in the effluents collected either in the jejunum or in the ileum before and after pea ingestion. The use of $^{15}$N-labelled pea protein allowed us to differentiate between the endogenous and exogenous N in the effluents. Before the ingestion of the test meal the basal flow rate of endogenous N was 0.22 (SD 0.15) mmol/min ($n$ 5) in the jejunum and 0.16 (SD 0.10) mmol/min ($n$ 7) in the ileum (Fig. 3). The endogenous N flow rate peaked significantly ($P < 0.05$) in the jejunum in the 40–60 min period, whereas no stimulation of the endogenous N could be detected in the ileum after meal ingestion.
The exogenous N was present in significant amounts ($P < 0.05$) in the jejunum during the 20–320 min period, peaking during the 40–60 min period and then decreasing rapidly, yet was present in significant amounts ($P < 0.05$) in the ileum during the 90–480 min period (Fig. 4). The ethanol precipitation, which gave an estimate of the balance between PN and NPN fractions of exogenous N, showed the ratios to be approximately 57:43 and 63:37 in the jejunum and in the ileum respectively.

**Kinetics of $[^{15}N]$pea nitrogen digestion and absorption**

The cumulative quantity of exogenous N that passed both at the proximal jejunum and at the terminal ileum was fitted according to a model curve in the form $y = b/[1 + \exp (c(t - a))] + d$ (Fig. 5). The curve reached a plateau value at 59.16 mmol in the jejunum and at 20.06 mmol N in the ileum. Taking into account the quantity of N ingested...
Fig. 3. The endogenous nitrogen fraction profiles in (a) the human jejunum and (b) the ileum. The endogenous nitrogen fractions were calculated from the difference between the total and the exogenous nitrogen fractions after \(^{15}\text{N}\)pea ingestion. Each value represents the mean of five subjects for jejunum and seven subjects for ileum; standard deviations are represented by vertical bars. The arrow represents the point of pea ingestion. *Mean values were significantly different from the basal value (t test, \(P < 0.05\)).

Fig. 4. The exogenous ethanol-soluble and -insoluble nitrogen fractions profiles in (a) the human jejunum and (b) the ileum. Ethanol precipitation of the effluents after \(^{15}\text{N}\)pea ingestion gave a supernatant fraction made up of peptides and amino acids (■) and a pellet fraction made up of proteins (▲). The exogenous nitrogen fractions were calculated from the \(^{15}\text{N},^{14}\text{N}\) ratio after \(^{15}\text{N}\)pea ingestion. Each value represents the mean of five subjects for the jejunum and seven subjects for the ileum; standard deviations are represented by vertical bars. The arrow represents the point of pea ingestion. *Mean values were significantly different from 0 (t test, \(P < 0.05\)).
Fig. 5. Cumulative exogenous nitrogen recovered in the jejunum (○) and the ileum (●) after [15N]pea ingestion. The experimental values of cumulative exogenous nitrogen recovered can be fitted to an exponential curve according to the equation \( y = \frac{b}{1 + \exp(c(t-u))} + d \) with \( a = 0.5, b = 47.6, c = -1.4 \) and \( d = -17.3 \) for jejunum and with \( a = 2.6, b = 11.8, c = -0.9 \) and \( d = -1.5 \) for ileum. Each value represents the mean of five subjects for the jejunum and seven subjects for the ileum.

Table 2. Estimation of nitrogen yield in the intestinal digesta after [15N]pea ingestion by healthy human subjects*

<table>
<thead>
<tr>
<th></th>
<th>Ingested (mmol N)</th>
<th>Recovered (mmol N)</th>
<th>Exogenous N absorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endogenous</td>
<td>Exogenous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Jejunum†</td>
<td>195</td>
<td>50.8</td>
<td>15.2</td>
</tr>
<tr>
<td>Ileum‡</td>
<td>195</td>
<td>39.7</td>
<td>6.4</td>
</tr>
</tbody>
</table>

* Exogenous and endogenous N fractions were calculated from the 15N:14N ratio after [15N]pea ingestion.
† N balance was calculated over 360 min.
‡ N balance was calculated over 480 min.

and the cumulative quantity of exogenous N recovered in each output compartment after pea ingestion, the present results show that the overall true gastro-ileal absorption of exogenous (pea) N was 89.4 (SD 1.1) % with 69 (SD 14) % of the exogenous N being absorbed between the stomach and the proximal jejunum (proximal absorption) and 20.4 % between the proximal jejunum and the terminal ileum (distal absorption) (Table 2).

**DISCUSSION**

Plant proteins, including pea protein, represent an interesting source of dietary protein in the human diet. Many studies have already dealt with the ability of soyabean to meet human N needs in short-term and long-term studies, but very little is known about pea.
protein (Young, 1991; Young & Pellet, 1994). The nutritional value of dietary protein for humans is usually determined from a 'chemical score' or more recently from the 'protein-digestibility-corrected amino acid score' in which digestibility represents an important aspect (Young & Pellet, 1991, 1994). This protein digestibility is usually obtained from human subjects using N balance studies (Food and Agriculture Organization/World Health Organization (FAO/WHO), 1990). In the present study the double-lumen tube technique was used to collect digesta either at the proximal jejunum or at the terminal ileum in order to quantify luminal movements of N and true pea digestibility after ingesting 75 g pea flour.

Before meal ingestion the basal flow rate of endogenous N was 13-4 (SD 12-0) mmol N/h (n 5) in the jejunum and 9-7 (SD 5-9) mmol N/h (n 7) in the ileum. These values are close to those previously obtained in human subjects (Mahé et al. 1994a). Souffrant et al. (1993) estimated the total endogenous flow of N in the pig terminal ileum to be approximately 6-8 mmol N/h. After pea ingestion the N recovered in the intestinal effluents originated from both exogenous (dietary) and endogenous (secreted) origins, but the use of 15N-labelled pea allowed us to differentiate between these two fractions at both the jejunal and ileal levels (Mahé et al. 1994b; Roos et al. 1994). The results indicated that the increase in N flow rate following [15N]pea ingestion both at the jejunal and ileal levels was mainly due to the dietary protein. A significant but slight increase in endogenous N secretion could be detected in the jejunum after pea ingestion. This observation agrees with both the idea that meal ingestion stimulates pancreatic secretion (Alpers, 1987) and with a previous study in which [15N]milk protein ingestion induced a significant increase in endogenous N at the jejunal level (Gaudichon et al. 1995). In contrast, no significant effect on endogenous N secretion could be observed at the jejunal level after the ingestion of small amounts of [15N]casein (Mahé et al. 1994b). Moreover, the results indicate that the endogenous N secreted in the jejunum was later reabsorbed before the ileal level where no increase in endogenous N could be detected.

The cumulative quantity of exogenous N, fitted according to a model curve in the form 

\[ y = \frac{b}{1 + \exp(c(t - a))} + d, \]

reached a plateau value of 20-06 mmol N in the ileum. This indicates that the digestion of pea N was completed after 8 h since the experimental recovery of exogenous N was 20-5 (SD 2-3) mmol N. The results confirm that the proximal intestine plays a major role in the digestion and absorption of protein since nearly 70% of the pea-protein N was already absorbed between the stomach and the 200 mm after the Treitz angle. These results resemble those obtained for soyabean protein (Baglieri et al. 1994) and also agree with the idea that gastric emptying represents an important step in the control of dietary N from protein with relatively high digestibility (Hara et al. 1992; Gaudichon et al. 1994). The distal part of the intestine is also important since it permits the absorption of 20% of the exogenous N and contributes to the reabsorption of endogenous N. Moreover, 35% of the total N recovered at the terminal ileum during the 8 h of the experiment was of exogenous origin. Undigested protein and unabsorbed peptides as well as amino acids, of both exogenous and endogenous origins, enter the colon and undergo digestion and metabolism by the microflora. Some products of this microbial metabolism may be either absorbed in the colon or excreted in the faeces and in the urine but are not of significant nutritional value (Rowan et al. 1994). The ethanol-insoluble fraction of the exogenous N at the terminal ileum remained constant at first, at a mean value of 59% during the 5-6 h following ingestion, and increased significantly (P < 0-05) to 75% after 360 min. This observation indicates the presence of an indigestible fraction in pea protein. In fact, pea protein is made up of a mixture of different fractions, including legumin and vicilin with different susceptibilities to proteolysis (Aubry & Boucrot, 1986). These results suggest that pea protein digestion is not homogeneous but involves (1) a fraction with high
and rapid digestion mainly absorbed in the proximal intestine, (2) a fraction more slowly absorbed in the distal intestine and (3) an indigestible fraction; these represent approximately 70, 20 and 10% of the protein respectively. Previous results with soyabean protein have also suggested a heterogeneous digestion of the protein (Tukur et al. 1993; Baglieri et al. 1994).

The present findings describe the gastro-ileal kinetics of exogenous N after pea ingestion. The 89.4% true ileal digestibility thus obtained for pea protein is in accordance with the 88% value for digestibility of mature pea proteins in man abstracted from balance experiments (FAO/WHO, 1990). Nevertheless, balance studies take into account neither the N absorbed in the colon (which leads to an overestimation of the digestibility) nor the endogenous secretions (which lead to an underestimation). The results obtained from balance studies are very close to those obtained in the present study but are devoid of true physiological significance. In animal studies the ileal digestibility of peas was previously reported to be 92%, whereas the apparent digestibility was only 74–79% (Huisman et al. 1992). This suggests that heat-treated pea protein has a good digestibility. Although the indigestible fractions of the pea protein and amino acids which pass through the large intestine are of limited nutritional value, these fractions could have a potential physiological role. Hence, further studies are now needed: (1) to estimate more precisely the nature of the indigestible fractions which pass through the ileo-caecal valve and (2) to measure the metabolic distribution of the dietary 15N absorbed.

The skilful assistance of M. Deyra from the gastroenterology unit of Avicenne Hospital (Bobigny, France) is gratefully acknowledged. The authors would like to acknowledge S. Salter for her English assistance.

REFERENCES


piglets fed on peas (Pisum sativum) and common beans (Phaseolus vulgaris). British Journal of Nutrition 68, 101-110.


Printed in Great Britain