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Identifying toxic fractions of wheat gluten and their effect on the jejunal mucosa in coeliac disease

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SUMMARY The toxicity of three fractions (A, B, and C) obtained by ultrafiltration of a peptic: tryptic digest of gluten has been assessed by serial feeding experiments in patients with treated coeliac disease.

The first fraction (A), which contains amino acids and oligopeptides, produced no damage to the jejunal mucosa.

The other two fractions (B and C) both caused mucosal damage.

Fraction B, which contains the products of digestion of smaller molecular weight, consists of polypeptides which are concentrated in the region of 8000 molecular weight. It contains no gliadin (molecular weight 50 000) or gluten.

Ultrastructural evidence of damage was visible six hours after challenge with fraction B and by 10 hours histological abnormalities were also present.

Ultrastructural abnormalities occurred early in the epithelial cells and preceded changes in the basement membrane and capillaries.

The disaccharidases showed a pronounced depression in all three subjects by 24 hours.

The rapid onset of damage after challenge, coupled with the evidence of recovery as soon as 72 hours later, is more in keeping with a direct action on the surface epithelial cells rather than an immune mechanism.

The exact component of gluten which is responsible for the mucosal damage in subjects with coeliac disease remains unknown. Wheat gluten is the residue obtained when wheat starch has been extracted from the dough made out of wheat flour. Gluten consists chiefly of glutenins and several ethanol-soluble α, β and γ gliadins (Ewart, 1970).

Crude gliadin or purified α-gliadin has been shown to be toxic in treated coeliac patients either by inducing malabsorption or by causing actual mucosal injury (van de Kamer, Weijers, and Dicke, 1953; Hekkens, Haex, and Willighagen, 1970; Kendall, Cox, Schneider, and Hawkins, 1972). It is not known whether the toxic properties of α-gliadin are a function of the whole molecule or reside in one or more of its component parts or whether the other gliadins are also toxic to subjects with coeliac disease.

Complete hydrolysis of gluten with dilute hydrochloric acid yields an amino acid mixture which is harmless to coeliac subjects (Alvey, Anderson, and Freeman, 1957). On the other hand, partial digestion of gluten with pepsin and trypsin yields a mixture (Frazer's fraction III) which retains the toxic properties of whole gluten (Frazer, Fletcher, Ross, Shaw, Sammons, and Schneider, 1959), as does a similar digest of crude gliadin (Krainick and Mohn, 1959; van Roon, Haex, Seeder, and de Jong, 1960; Bronstein, Haeffner, and Kowlessar, 1966).

This paper describes further attempts to identify the toxic component of gluten starting with Frazer's fraction III.

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Materials and Methods

PREPARATION OF FRACTIONS OF GLUTEN

The initial digestion procedure followed was similar to that used by Frazer, Fletcher, Ross, Shaw, Sammons, and Schneider (1959). It was noted that a precipitate was formed when the peptic : tryptic digest of gluten was kept in the cold. This precipitate probably consisted of undigested gluten or gliadin and it was removed by centrifugation.

Separation into further fractions was made on the basis of molecular size. Because of the relatively large amounts which we decided to use for feeding experiments, column chromatography methods of separation were judged to be unsuitable and it was decided to employ ultrafiltration. This was carried out in the cold using an Amicon 2-litre cell and an oxygen-free atmosphere. The first fraction (fraction A) was an ultrafiltrate obtained from the peptic: tryptic digest using a UM 10 membrane (Amicon Ltd). Since the exclusion limit of this membrane is 10 000 (molecular weight), the ultrafiltrate could be said to contain only electrolytes, amino acids, and small peptides not exceeding a molecular weight of 10 000.

The residue was a thick, syrupy liquid which was then resuspended in distilled water and passed through a second membrane which was an XM100a membrane (Amicon Ltd). The ultrafiltrate obtained was a clear, serum-like fluid which was called fraction B. The residue remaining in the ultrafiltration chamber after this second filtration was called fraction C. This last fraction was not completely soluble in water.

Each fraction was freeze-dried. The yield of fraction A from 1000 ml of the peptic : tryptic digest of gluten was less than 5 g, that of fraction B was about 15 g, and that of fraction C was about 30 g. For preparation of a sufficiently large supply of each fraction, several kilograms of gluten had to be digested.

CHARACTERIZATION OF FRACTIONS

Physical characteristics

Fraction A is a colourless ultrafiltrate which tastes of salt. In the freeze-dried form, it is a white fluffy powder. The pH of fraction A dissolved in distilled water is 5·2. The electrical conductivity is high (26 m^{-1} cm^{-1}). This is because there is much ionic material in this fraction as most of the NaCl produced in the digestion procedure appears in this ultrafiltrate.

Fraction B is a clear, pale yellow, serum-like fluid with a slightly flour-like taste. In the freeze-dried form it is a yellowish white, crystalline powder. This powder is completely soluble in water in quite high concentrations, eg, 100 mg per ml. The pH of the aqueous solution is 4·2. The electrical conductivity is 5·5 m^{-1} cm^{-1}.

Fraction C, the residue from the second ultrafiltration, contains insoluble material and even after freeze-drying this greyish white powder does not dissolve completely. Its aqueous solution has a pH of 4·5. The electrical conductivity is 1·7 m^{-1} cm^{-1}.

Nitrogen content

The nitrogen content estimated by the Kjeldahl method is as follows: fraction A 4·6 mg%; fraction B 12·8 mg%; fraction C 14·7 mg%. That of pure gliadin is about 16 mg% (Peña Ramirez, 1973).

Amino acid composition

This was determined by dissolving 10 mg of each freeze-dried fraction in 2 ml of a solution containing concentrated HCl (Aristar R): H_2O in equal proportions and 1% phenol. Of this, 0·2 ml was placed in an acid-washed tube and sealed under nitrogen. The tubes were placed in an oven at 808°C for 24 hours. After hydrolysis the samples were dried down and analysed in a Beckmann autoanalyser. Duplicate samples of each fraction were analysed in this way. Very little free amino acid occurs in fractions A, B, or C. The amino acid composition of each fraction after complete hydrolysis demonstrated that there are no qualitative differences in the amino acid compositions of the various fractions but only slight differences in the ratios of the percentage content of one amino acid to another.

Paper chromatography and electrophoresis

This was carried out in a standard manner (Gonzalez and Offord, 1971) and showed that fraction A contains free amino acid and some fast-moving peptides.

Fraction B showed a large number of ninhydrin-positive spots and neutral as well as acidic and basic peptides. There were no large peptides or whole proteins in the fraction.

Fraction C also contained a mixture of peptides, some of which correspond to those in fraction B but there were more slow-moving peptides, probably of a larger molecular weight.

Polyacrilamide gel electrophoresis

Sodium dodecyl sulphate polyacrilamide gel electrophoresis was performed on fractions A, B, and C as well as on gluten according to the method of Weber and Osborn (1969). The electrophoresis was done using 25 and 400 µg of each of the test substances. No bands were visible with fraction A even when used in the higher concentration. Fraction B showed a single fast band which corresponds to a molecular weight of about 8000. There is a tailing effect to the band suggesting the presence of slightly larger peptides. Fraction C also showed a single band but one corresponding to a molecular weight of about 13 000. Neither fraction B nor fraction C showed any bands corresponding to gliadin or gluten even when used in high concentrations. Therefore the conclusion appears to be that these fractions contain no appreciable amounts of undigested gliadin.

Gel electrophoresis has also been performed by the method of Narayan, Vogel, and Lawrence (1965) and this confirmed the absence of whole gliadin in the fractions (Peña Ramirez, 1973).

ASSESSMENT OF TOXICITY

Toxicity was tested by observing the effect of the three fractions on the jejunal mucosa of patients with treated coeliac disease. First it was necessary to undertake a pilot study in order to determine a suitable dose and if one or all the fractions would produce an effect. Three patients (J.S., R.P., and V.B.) adhering to a strict gluten-free diet volunteered to take part. At the start of the experiment the jejunal biopsy appearances in all three had virtually returned to normal. The patients were given a supply of the freeze-dried fractions, each day's dose being in a separate bottle. They were asked to take the powder dissolved in water as a drink with their meals or to mix it with the food. The first patient (J.S.) was given a dose of 5 g/day of each fraction, the second patient (R.P.) 10 g/day, and (V.B.) 20 g/day.

Each patient took fraction A for one week, and then a second biopsy was performed. This proved to be normal and so fraction B was taken for one week and a further biopsy was performed. The mucosa in all three patients was abnormal. The damage appeared to be dose related, the greatest abnormality being in V.B. A strict gluten-free diet was kept for three to four weeks in order that the mucosa could recover. A check biopsy was performed showing that this in fact had occurred and fraction C was given for one week followed by a further biopsy. Abnormalities were again observed and once more appeared to be dose related.

Fraction A therefore did not appear to be toxic whereas both B and C were, and because fraction B contained the smaller molecules this was chosen in order to investigate its effect on the mucosa more thoroughly. Six months later two of the original three patients (J.S. and R.P.) volunteered again and were joined by E.C. All had been on a gluten-free diet and had normal features on jejunal biopsy at the start of the experiment. Blood was taken for haematological examination and immunoglobulin estimation. Fraction B dissolved in distilled water was given by mouth. Patients J.S. and R.P. were given 20 g three times a day up to a total of eight doses and seven doses respectively. E.C. had a single dose of 40 g. Jejunal biopsy was performed with a Crosby-Kugler capsule as modified by Salem, Salt, and Truelove (1965). They were taken under radiological control 5 to 10 cm beyond the ligament of Treitz. Biopsies were taken at the following times after the start of the experiment: patient J.S. 6, 24, 48, and 72 hours; patient R.P. 10, 24, 48, and 72 hours; patient E.C. 10, 24, and 72 hours. Each biopsy was divided into three parts and handled as follows:

1 One piece was fixed in 10% formalin for light microscopy intraepithelial lymphocyte counting and quantitative histology by the method of Dunnill and Whitehead (1972).

2 A second piece was processed for electron microscopy.

3 The remainder of the biopsy specimen was wrapped in parafilm and kept at −20°C for disaccharidase estimations. The disaccharidases were estimated by the procedure of Burgess, Levin, Mahalanabis, and Tonge (1964). Disaccharidase activity was expressed as units per gram wet weight (Peña Ramirez, 1971).

Results

CLINICAL FEATURES

All three patients were symptom-free at the time of entering the study. Patient J.S. remained entirely symptom-free throughout the three days and also after he had returned to being on a strict gluten-free diet. Patient R.P. noticed no symptoms while he was taking the fraction B, but symptoms started about the fourth day and persisted for over three weeks after the challenge. The symptoms were mild and consisted of a feeling of malaise, occasional upper abdominal discomfort, and mild diarrhoea with two to three motions a day. The appearance of the faeces was suggestive of steatorrhoea and his weight fell by about 2 kg. Patient E.C. first noted symptoms about five hours after taking the large single dose of fraction B. She began to experience generalized abdominal discomfort and distension and vomited once. She also had tachycardia and appeared mildly shocked. However, these symptoms had passed off by 24 hours after challenge and she has remained symptom-free since.

HAEMATOLOGICAL INVESTIGATIONS

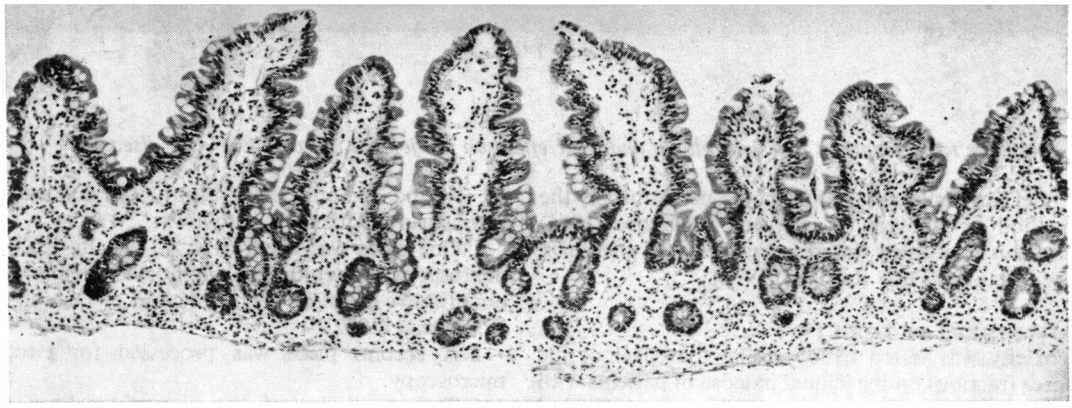
No consistent changes occurred in any of the routine blood investigations. No changes were observed in serum immunoglobulins, apart from a fourfold increase in IgM after challenge in one patient (R.P.).

BIOPSY FEATURES

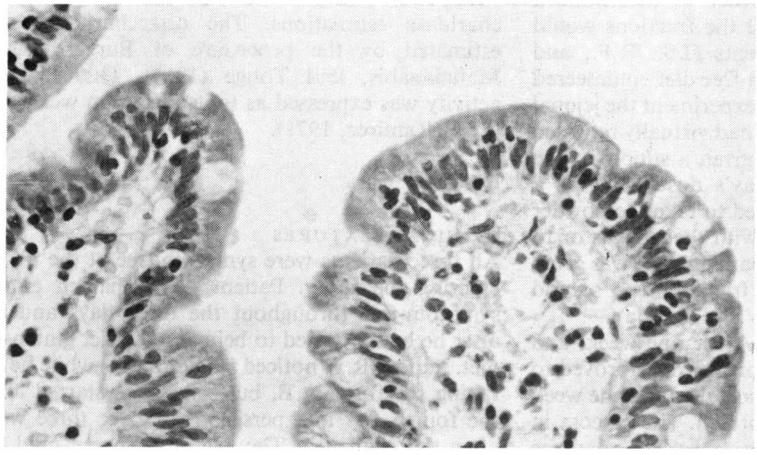
Light microscope appearances

The changes are illustrated in figs 1A to D which were prepared from the biopsies taken from patient E.C. In all three patients the initial jejunal biopsy specimen showed normal appearances or only minor abnormalities such as a slight distortion of the villous architecture and a small excess of inflammatory cells in the lamina propria (fig 1A 1 and 2). Histological changes were already established at

A1



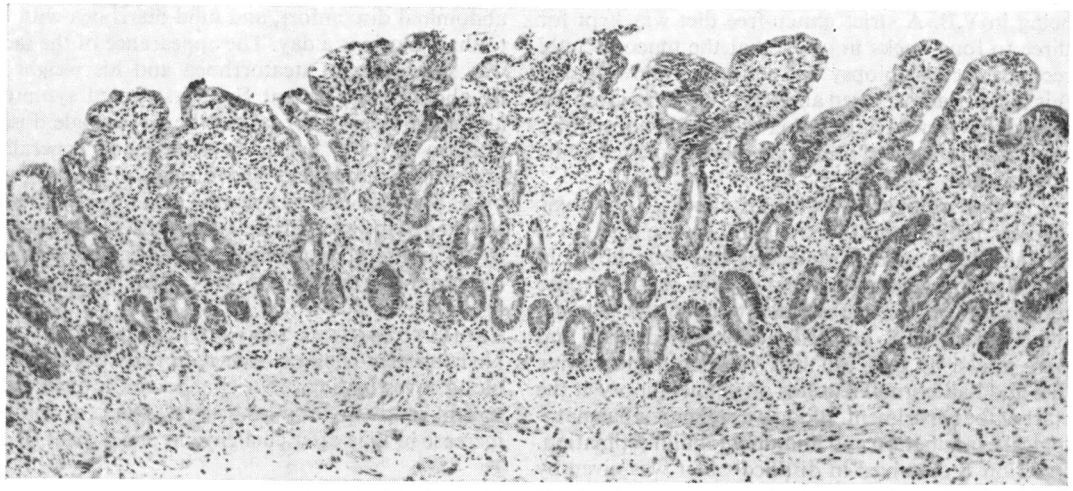
A2



Figs 1 A to D *Histological changes observed at low (1) and high (2) power on serial jejunal biopsy of patient E.C. who received a single dose of fraction B.*

A *1 and 2 Immediately before consuming fraction B.*

B1



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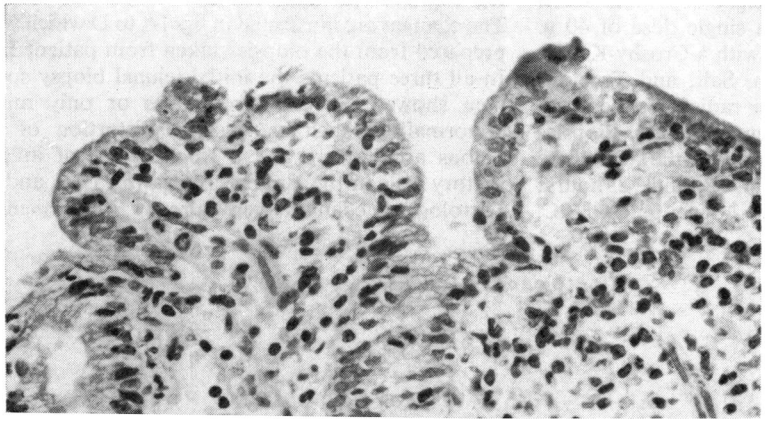
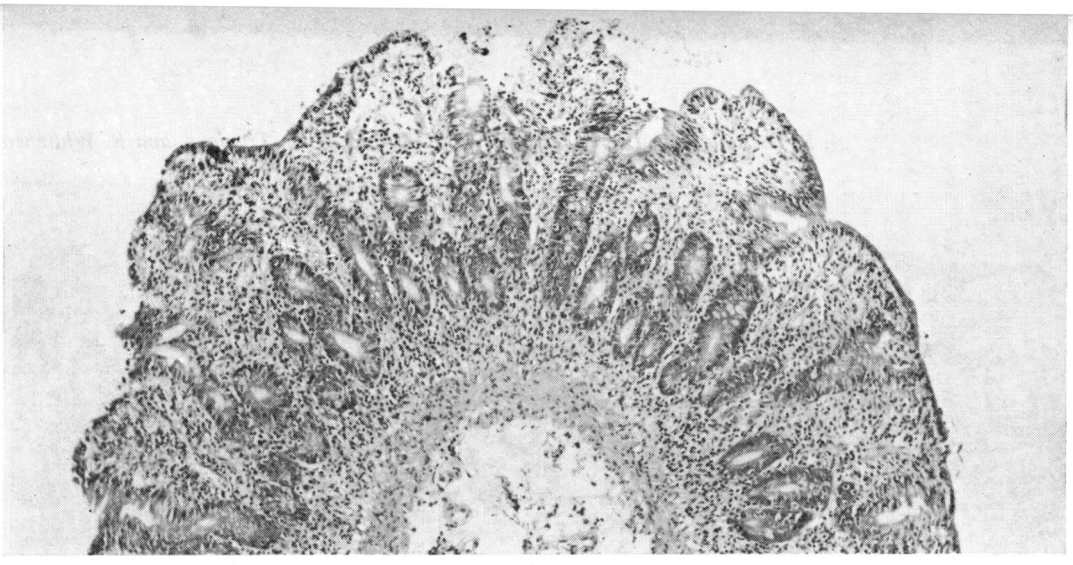
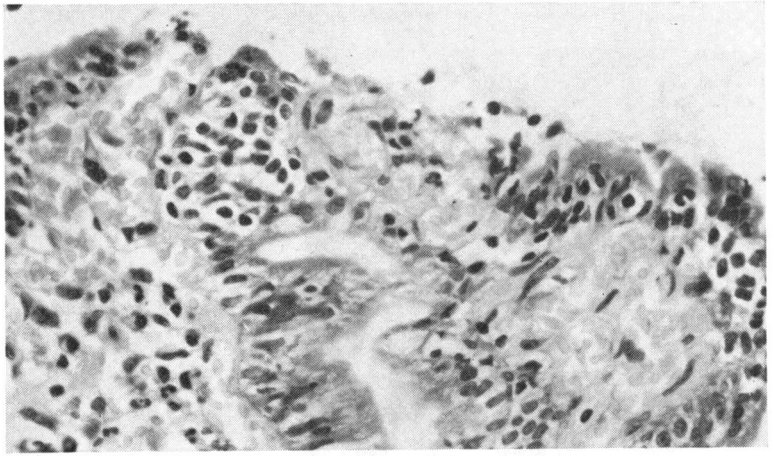


B *1 and 2 At 10 hours.*

C1





C2

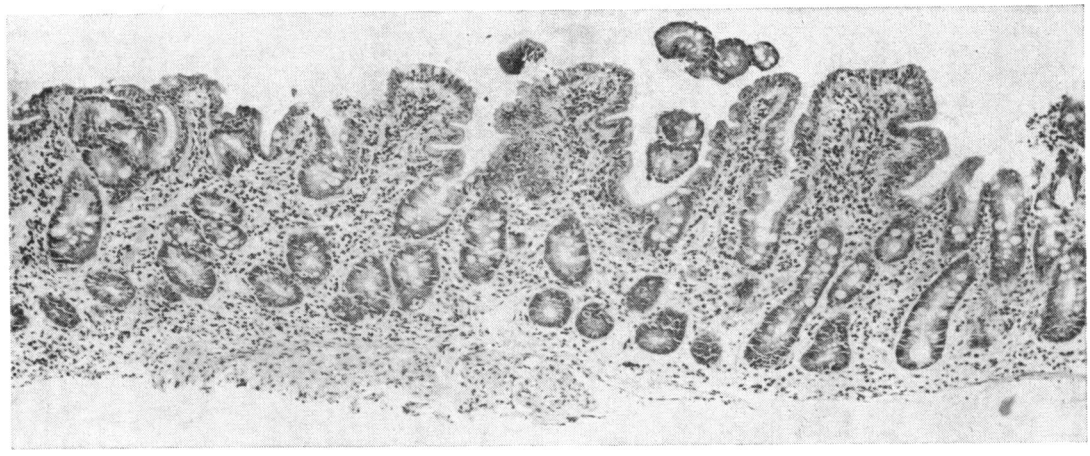
C 1 and 2 At 24 hours.

10 hours. The villous architecture had become markedly abnormal, the appearances being those of severe partial villous atrophy (fig 1B 1 and 2). The surface epithelial cells were cuboidal and showed nuclear palisading. The brush border appeared irregular and fuzzy. There was a marked increase in intraepithelial lymphocytes and numerous polymorphs were also present. Lymphocyte and polymorph infiltration of the lamina propria was marked. In the case of the 10-hour biopsy from E.C. (who received a single large dose) an additional feature was the presence of microerosions in the epithelium. At this stage alterations in the numbers of crypt cell mitotic figures were inconstant but Paneth cells were reduced in number and showed varying degrees of degranulation.

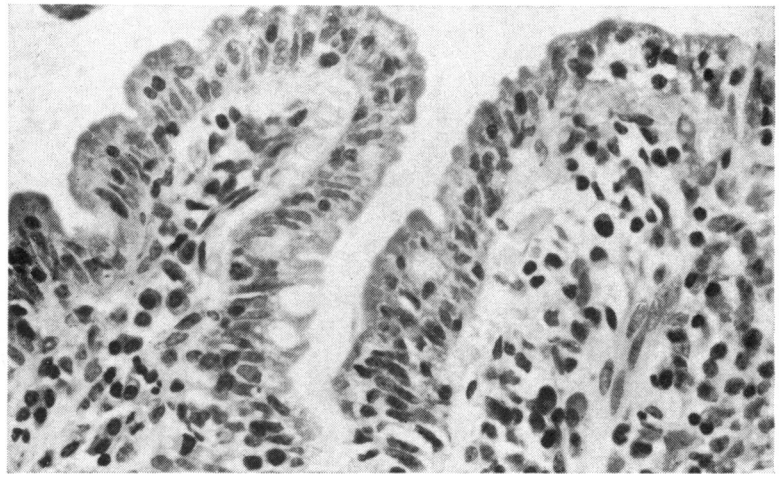
By 24 hours the biopsy specimens from all three patients showed very severe changes similar to those seen in biopsy specimens from patients with untreated coeliac disease (fig 1C 1 and 2). In the case of the two patients who received repeated doses of fraction B, similar changes were persisting at 48 and 72 hours. However, in the third patient who received a single dose of fraction B evidence of recovery was present in the biopsy specimen taken at 72 hours (fig 1D 1 and 2). Villi were beginning to reform, although they were short and distorted; the surface epithelial cells were becoming columnar and the nuclear arrangement was more regular; and there were fewer intraepithelial lymphocytes and hardly any polymorphs. Paneth cells were more or less normal in number and in appearance.

Surface : volume ratio

The two patients who received repeated doses of fraction B showed a fall in the surface: volume ratio which became apparent at 10 hours after the first dose and then persisted for the remainder of the period of study. The other patient, who was given a single large dose, showed a progressive fall in this ratio over the first 24 hours, at which time the value was severely abnormal and was in the range associated



D1



D2

D *1 and 2 At 72 hours.*

with untreated coeliac disease (Dunnill and Whitehead, 1972). However, by 72 hours the surface:volume ratio was already rising towards the normal range (table I).

Time (h) after Fraction B	*J.S.*[1]	*R.P.*[1]	*E.C.*[2]
0 control	52·5	55·7	65·3
6	54·3	—	—
10	—	35·3	31·8
24	43·1	35·4	12·7
48	29·9	38·6	—
72	27·1	40·1	34·8

Table I *Surface: volume ratio of serial biopsy specimens after challenge with gluten fraction B*

[1]These two patients received 20 g fraction B three times a day for 60 hours (J.S.) and 54 hours (R.P.) respectively.
[2]This patient received a single dose of 40 g fraction B immediately after the first biopsy.
Normal surface: volume range 46·0 (sd 12·0) (Dunnill and Whitehead, 1972).

Intraepithelial lymphocytes

The results of the count in the serial biopsies from the three patients studied are shown in table II. These results provide objective evidence of a genuine

	Hours					
	0	*6*	*10*	*24*	*48*	*72*
J.S.[2]	1440	2192	—	2800	4492	6000
R.P.[2]	2190	—	4440	3557	3245	3520
E.C.[3]	2147	—	3530	4580	—	2890

Table II *Intraepithelial lymphocyte counts in serial biopsy specimens after challenge with fraction B*[1]

[1]The counts were made by a modification of the quantitative method of Dunnill and Whitehead (1972) and are based on the number of lymphocytes in five microscope fields and expressed as the number per 100 epithelial intercepts.
[2]These two patients received 20 g fraction B three times a day for 60 hours (J.S.) and 54 hours (R.P.) respectively.
[3]This patient received a single dose of 40 g of fraction B immediately after the first biopsy.

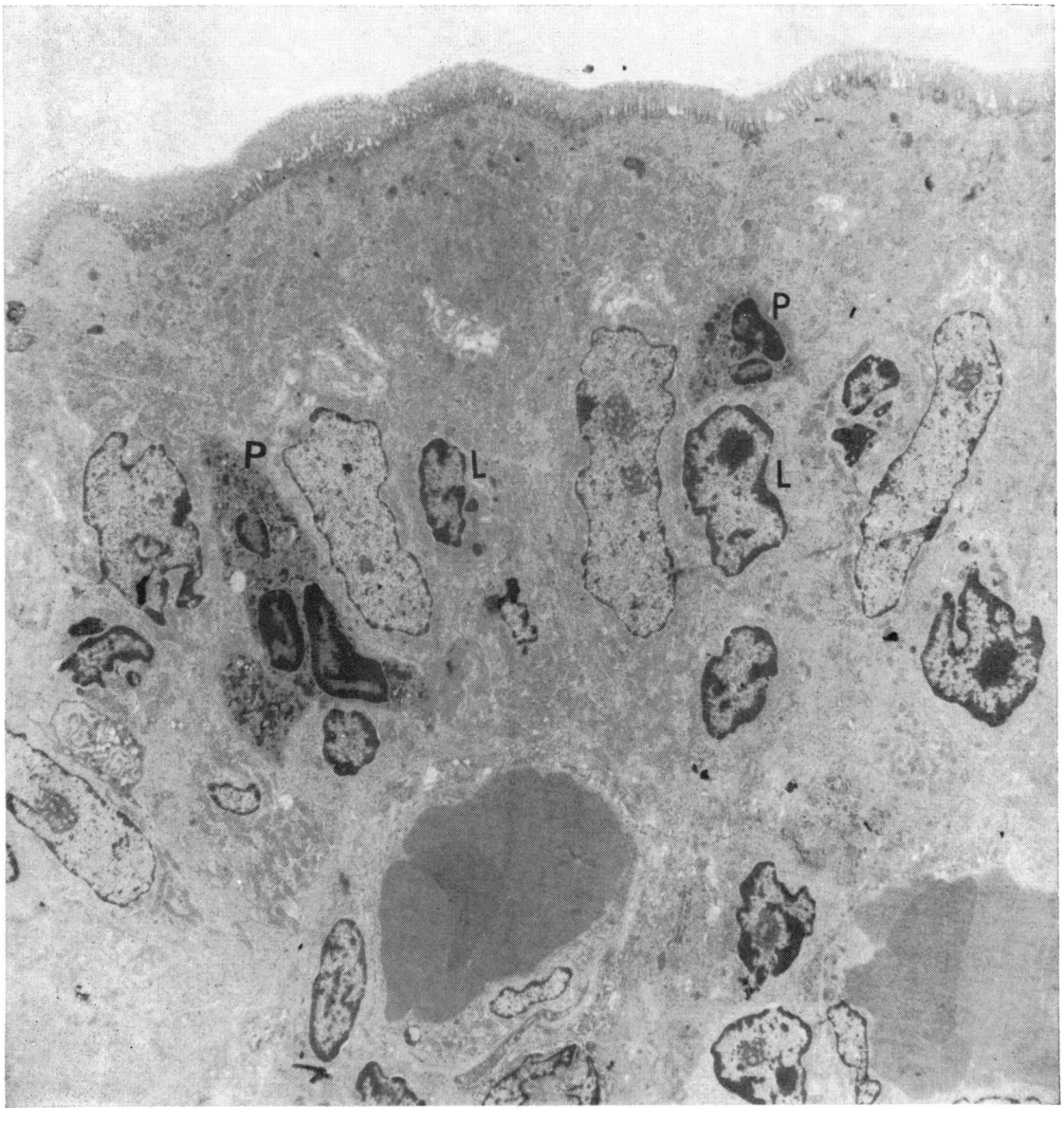


Fig 2 A *Surface epithelium at six hours. Lymphocytes (L) and polymorphs (P) between the epithelial cells (× 4200).*

Figs 2 A to G *Some ultrastructural changes after ingestion of a single dose of gluten fraction B.*

increase in intraepithelial lymphocytes. The marked increase in the count in the six-hour biopsy specimen is compatible with the view that an effect on the mucosa had already begun by this time.

Ultrastructural features (figs 2 A to G)

The initial biopsy specimens which were taken immediately before challenge with fraction B showed no abnormal appearance with the sole exception that

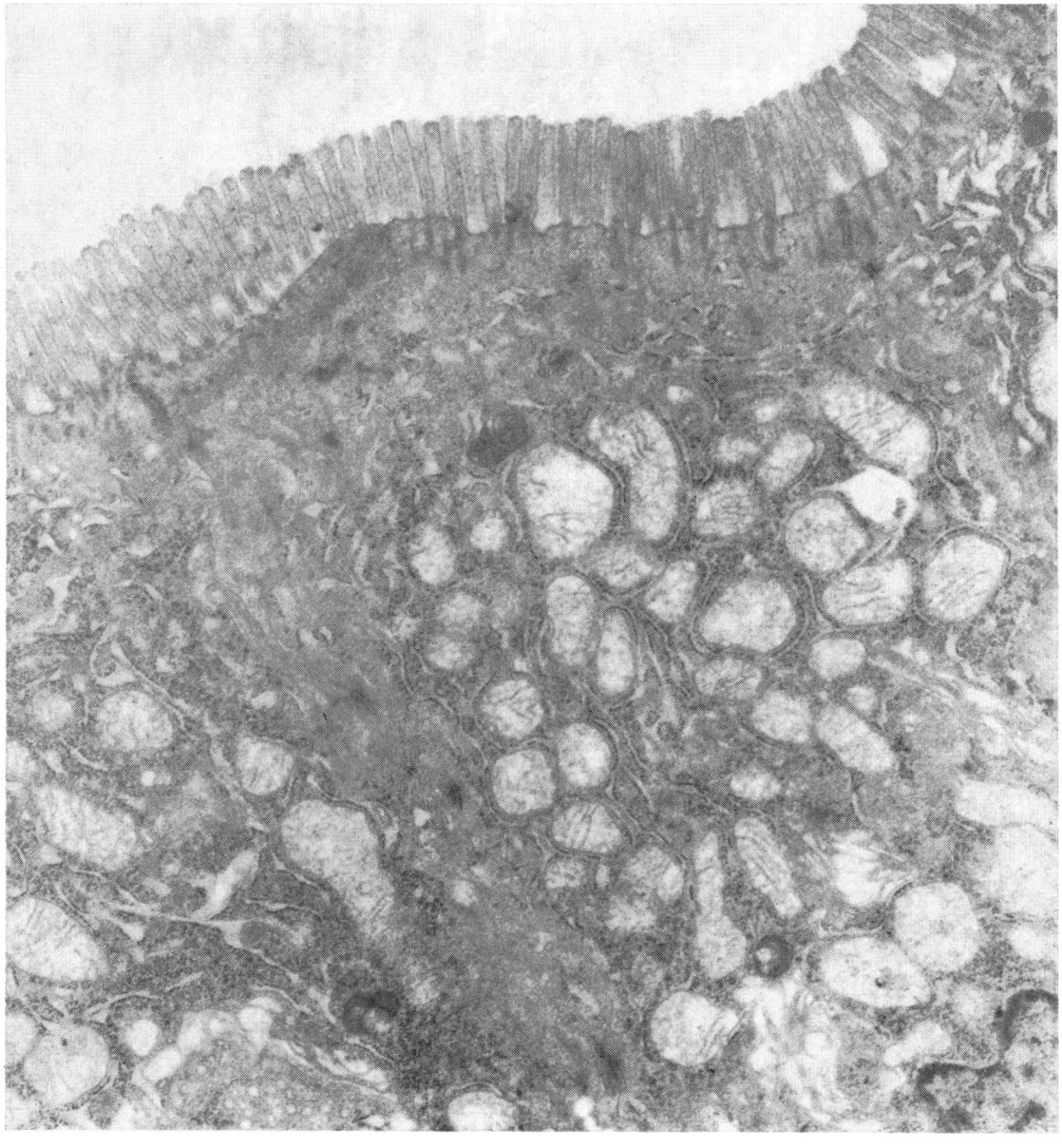


Fig 2 B *Surface epithelium at 10 hours. Severe mitochondrial swelling, pallor, and fragmentation of cristae* (× *7400*).

in the case of J.S. there were some areas in the sub-epithelial basement membrane with an excess of connective tissue fibrils.

In the biopsy specimen taken at six hours there was some intercellular oedema. The microvilli of the surface epithelial cells were normal but lysosomal bodies appeared to be more frequent and there was swelling of the mitochondria with irregularity and disruption of their cristae so that some appeared distinctly pale and vacuolated. Similar abnormalities were also observed in the crypt cells. There was an increase in the cells in both the lamina propria and within the surface epithelium and polymorphs were prominent among these cells. However the basement

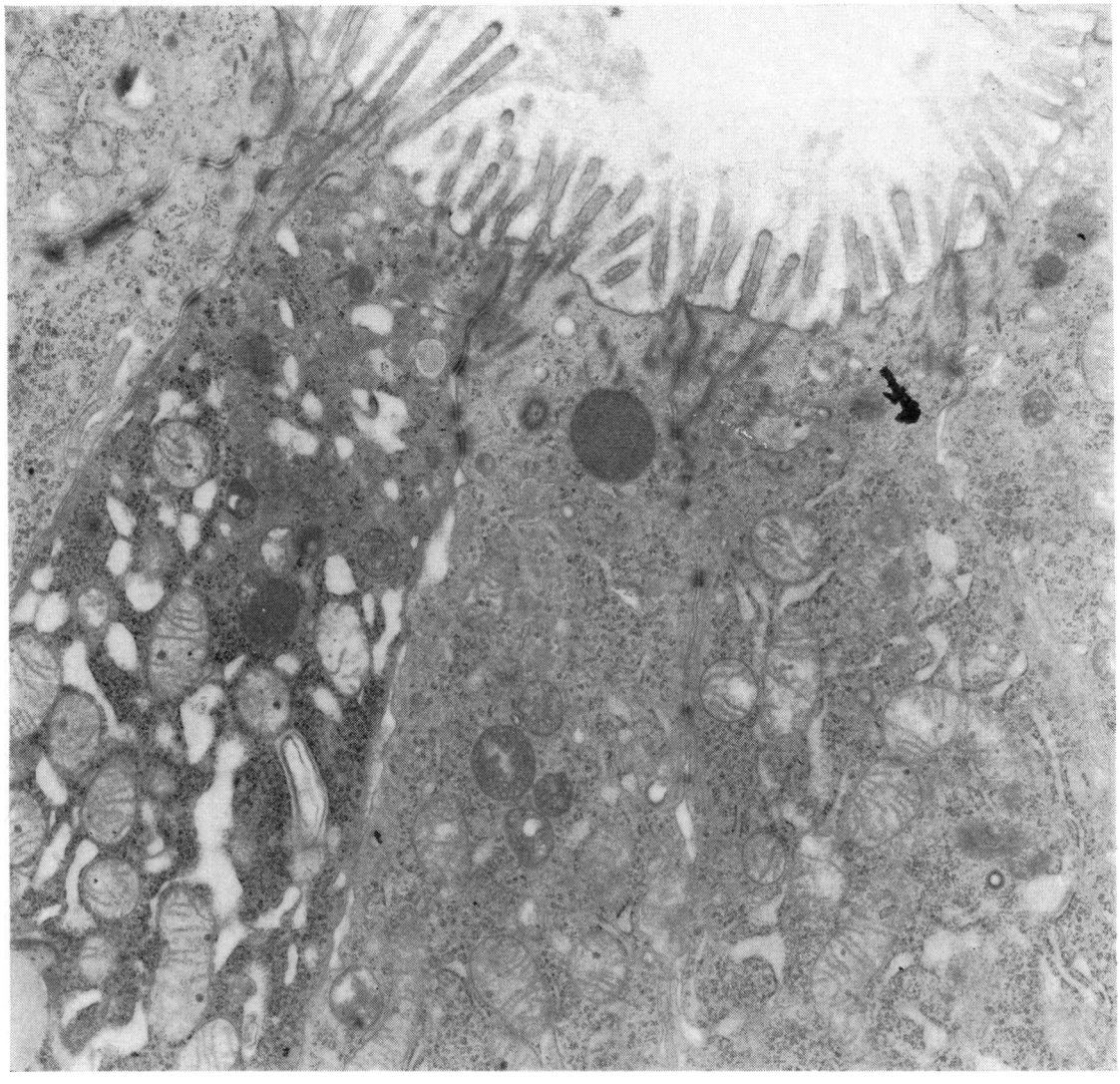


Fig 2 C *Crypt epithelium at 24 hours. Mitochondrial damage present here also. Note lysosomes and multivesiculate bodies* (× *17 400*).

membrane did not appear to be abnormal and the capillary endothelium showed no evidence of hyperplasia although the capillary basement membrane was thickened in some places.

By 10 hours there were more pronounced epithelial cell changes. The principal abnormalities were shortening and irregularity of the arrangement of the microvilli; mitochondrial degeneration and an increase in lysosomal and multivesiculate bodies and in the polyribosomes. Once again a striking feature was the presence of polymorphs within the surface epithelium between the epithelial cells.

At 24 hours the abnormalities were more pronounced and in addition there was thickening of the basement membrane of the absorptive epithelium which had also acquired a certain fibrillary quality. The endothelial cells of the subepithelial capillaries were also plumper and their intraluminal processes markedly hypertrophied. There was also conspicuous thickening of the capillary basement membrane.

In the 48- and 72-hour biopsies from patients J.S. and R.P. similar features were present but the

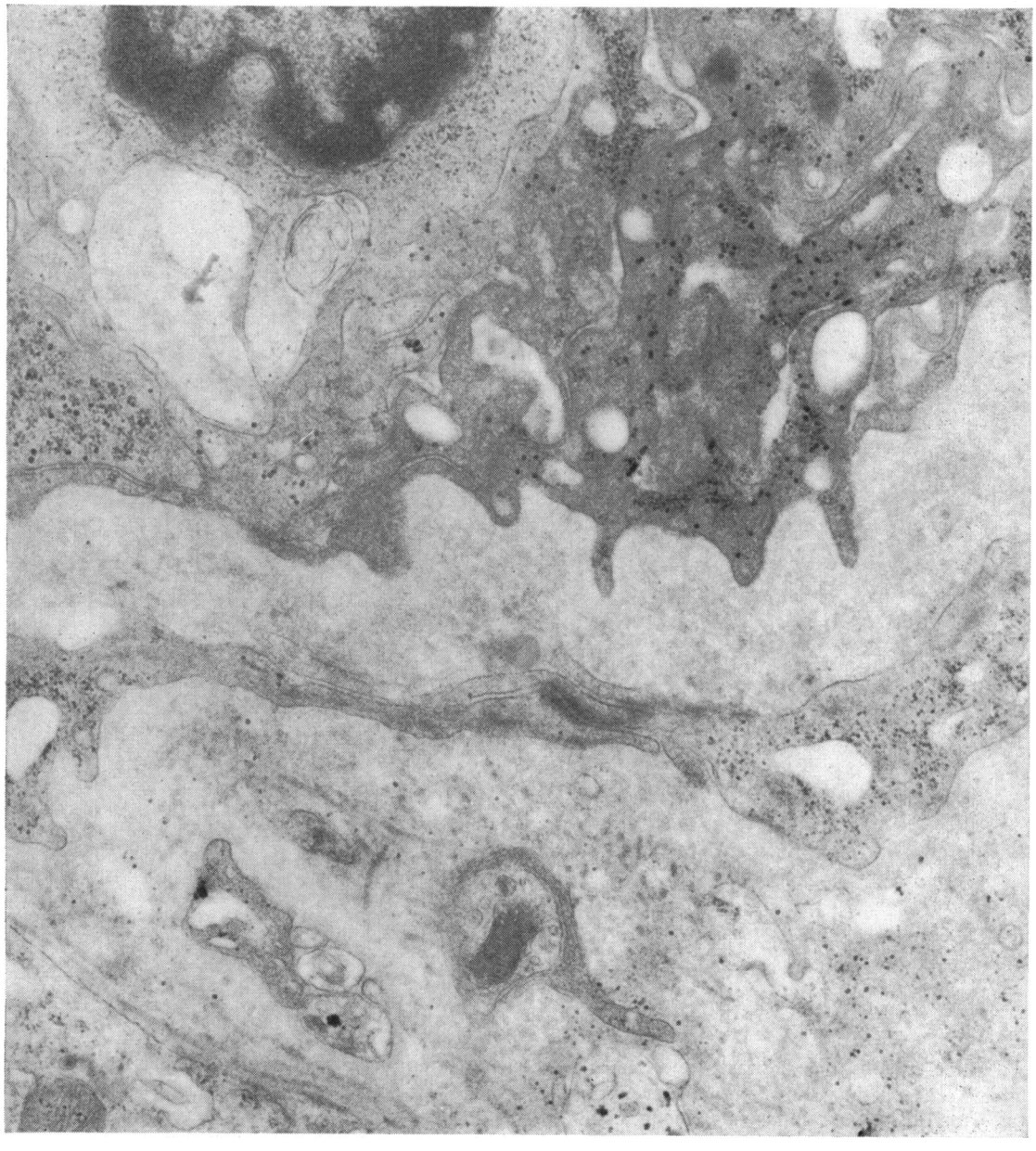


Fig 2 D *Surface epithelial basement membrane at 24 hours. It is thicker than normal and shows early fibril deposition (× 32 400).*

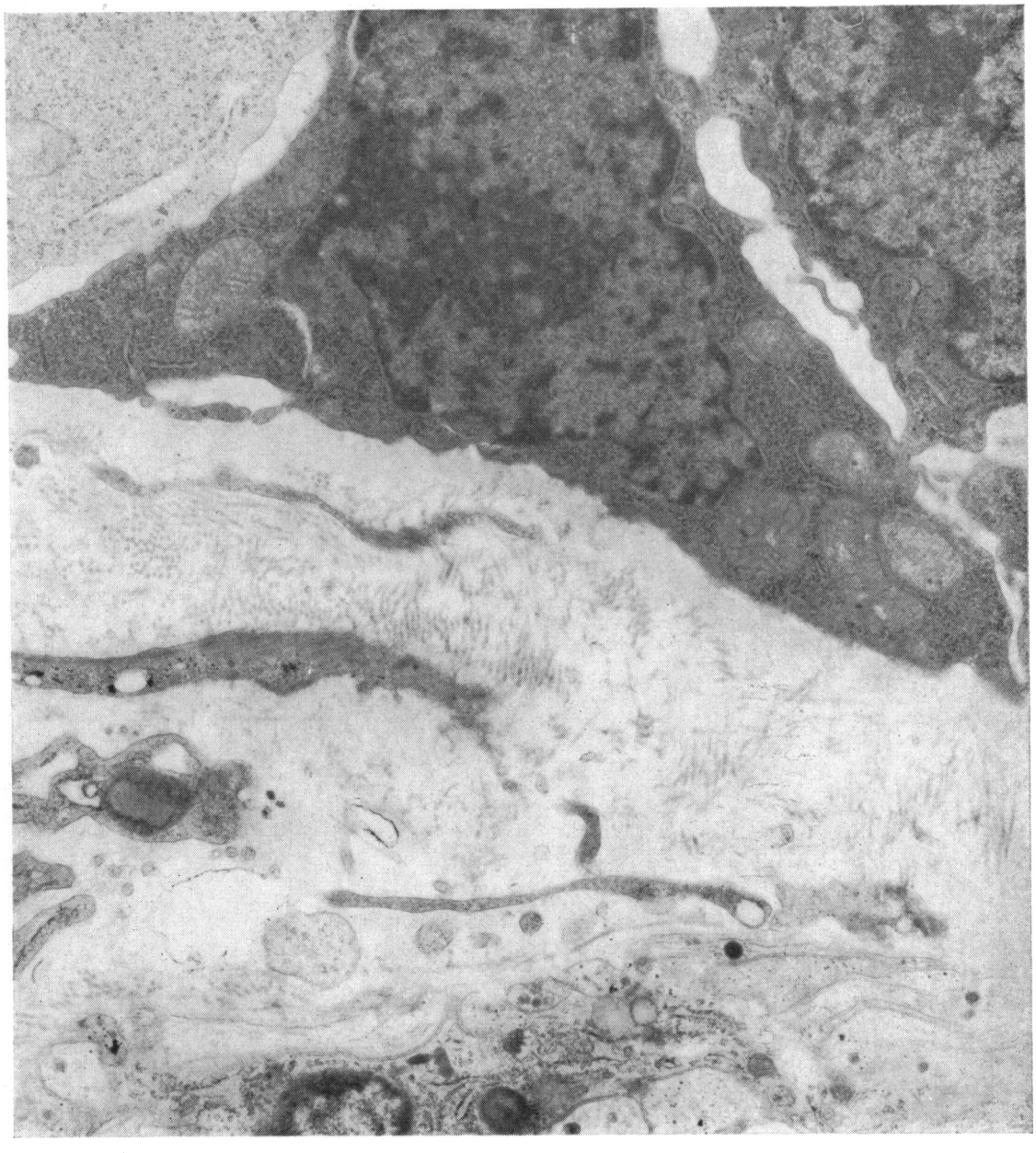


Fig 2 E *Surface epithelial basement membrane at 72 hours. The abnormal fibril component is now obvious* (× *17 400*)

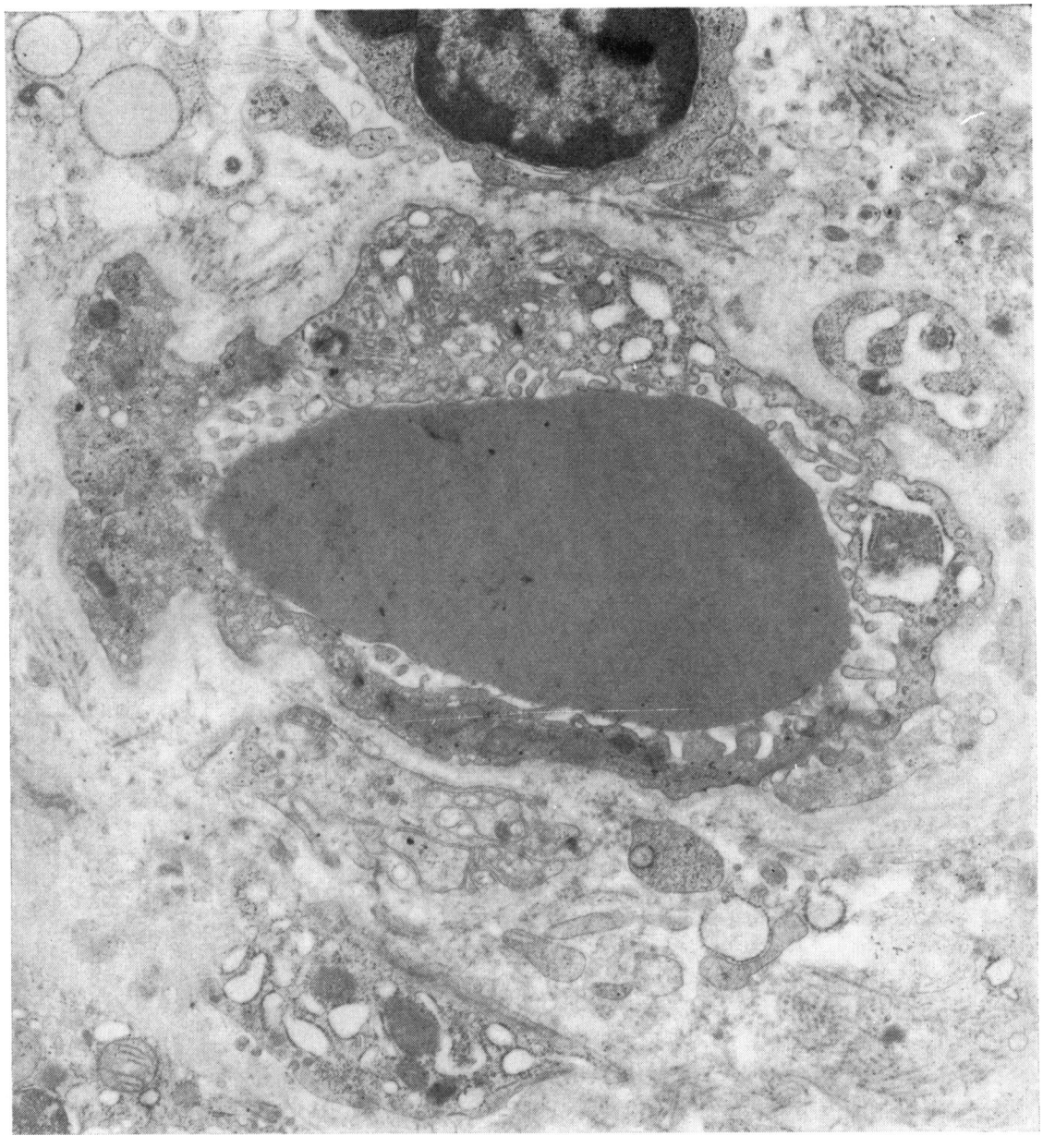


Fig 2 F *Capillary in lamina propria at 24 hours. There is thickening of and early fibril formation in the basement membrane. The endothelial cell shows hyperplasia with increased bulk and complexity of luminal processes* (× *17 400*).

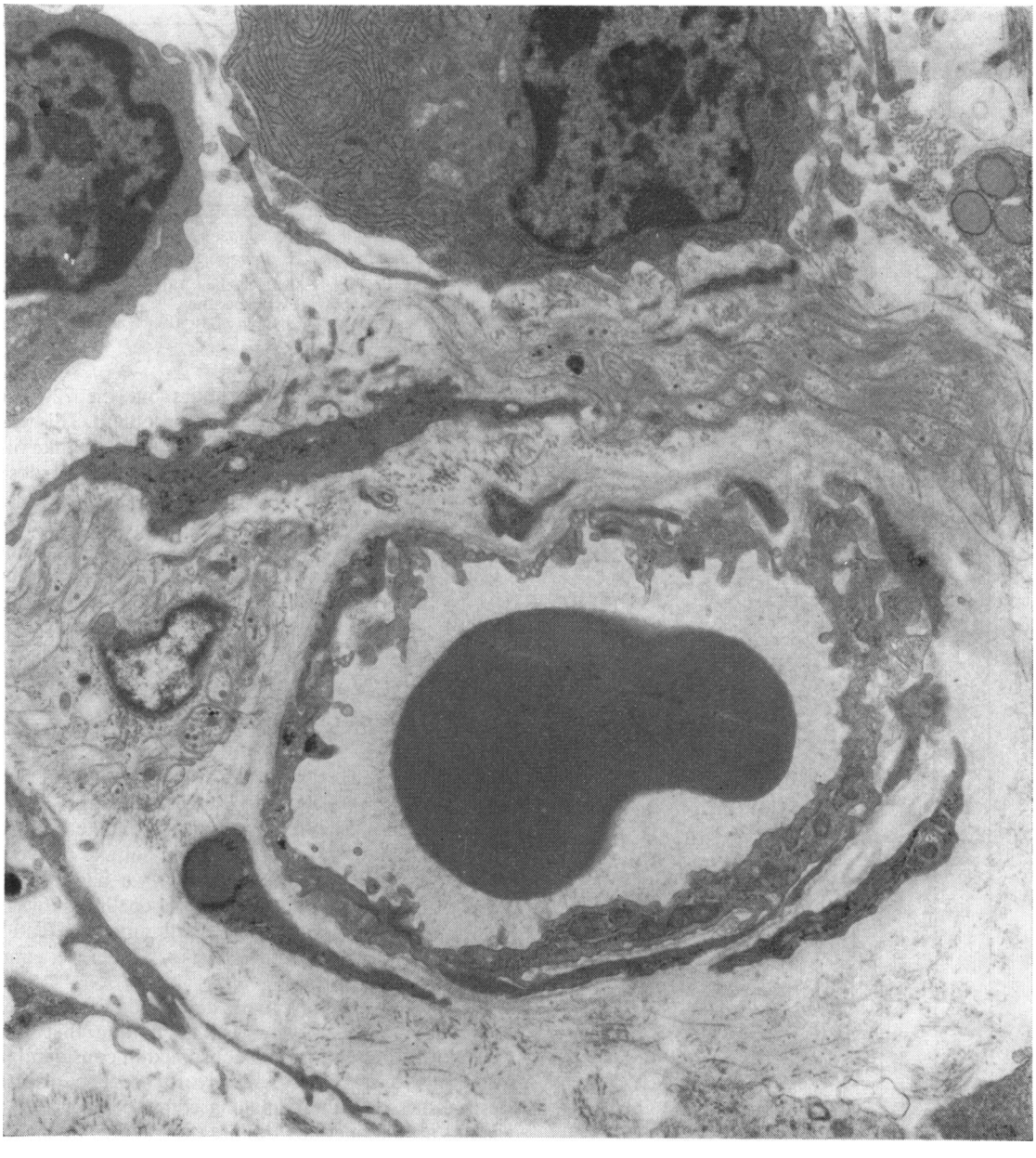


Fig 2 G *Capillary in lamina propria at 72 hours. Fibril component of the thickened basement membrane is more obvious* (× *10 800*).

thickening of the absorptive cell basement membrane and capillary endothelial hypertrophy and basement membrane thickening were more pronounced.

JEJUNAL DISACCHARIDASES

In all three patients the disaccharidases in the initial biopsy specimens were all within normal limits. The subsequent changes are shown in table III.

With patient J.S. no change in the enzyme content was noted at six hours but by 24 hours a precipitous fall had occurred with all three enzymes, which continued to fall over the next two days.

In patient R.P. the jejunal disaccharidases were reduced at 10 hours and fell further by 24 hours.

Disaccharidase /g Wet Weight	*Patient*	*Hours after Challenge*					
		0	*6*	*10*	*24*	*48*	*72*
Lactase	J.S.[1]	1·8	2·1	—	0·7	0·4	0·4
	R.P.[1]	3·3	—	1·9	0·8	0·4	0·8
	E.C.[2]	4·4	—	0	0	—	0·3
Sucrase	J.S.[1]	7·2	7·8	—	4·2	3·1	3·1
	R.P.[1]	8·6	—	4·2	3·1	2·2	2·8
	E.C.[2]	6·9	—	1·7	0·6	—	1·1
Maltase	J.S.[1]	23·9	23·1	—	15·3	11·7	13·3
	R.P.[1]	26·7	—	21·8	14·4	5·8	11·7
	E.C.[2]	26·1	—	4·7	0·6	—	3·3

Table III *Jejunal disaccharidases in serial jejunal biopsy specimens after challenge with gluten fraction B*

[1]These two patients received 20 g fraction B three times a day for 60 hours (J.S.) and 54 hours (R.P.) respectively.
[2]This patient received a single dose of 40 g fraction B immediately after the first biopsy.

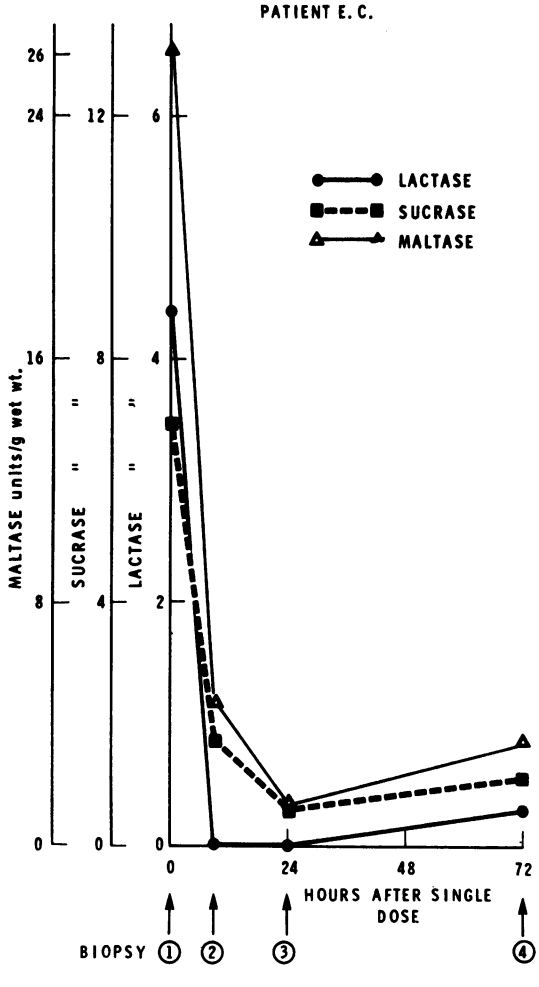


Fig 3 *Disaccharidase activity of serial jejunal biopsy specimens obtained from E.C. after a single dose of fraction B.*

They remained low at 48 hours but the 72-hour biopsy showed some rise in enzyme content. This was almost entirely because this patient failed to take his last dose of fraction B and therefore the last dose administered was 18 hours before the biopsy.

A similar but more marked fall occurred after nine hours with a single large dose of fraction B (patient E.C.). The enzymes were even lower at 24 hours but by 72 hours there was evidence of enzyme recovery (fig 3).

Discussion

The pilot experiment produced no evidence to suggest that fraction A was toxic. There remains the possibility that the dosage of fraction A used was insufficient to produce any mucosal damage. However, this is most unlikely as a daily intake of 20 g of fraction A corresponds to a daily intake of 400 g of gluten; and, in another study, we have found jejunal mucosal abnormalities in coeliac subjects who were consuming very small amounts (<0·5 g/day) of gluten (Dissanayake, Truelove, and Whitehead, 1974).

Fraction A contains amino acids and oligopeptides (<1000 molecular weight). The finding that this fraction is not toxic when consumed in considerable amounts by patients with treated coeliac disease establishes that the damaging component of gluten is not an oligopeptide despite the findings of earlier workers (Krainick and Mohn, 1959; Bronstein *et al*, 1966), and supports the later work of Douglas and Booth (1970) and Berg, Dahlqvist, Lindberg, and Nordén (1970).

Both fraction B and fraction C have been found to damage the jejunal mucosa in coeliac subjects. Fraction B contains the smaller molecules and the eventual aim of this study is to determine the smallest

toxic component of gluten. Fraction B is a heterogeneous mixture of peptides but they are concentrated in the region of a molecular weight of 8000. Much further work remains to be done to separate its component peptides and to assess their individual toxicities, but it can be said with certainty that it contains no gliadin (molecular weight about 50 000) and no gluten.

Damage to the small intestinal mucosa starts within six hours of feeding fraction B by mouth and is pronounced by 10 hours. The toxicity is also confirmed by the steep fall in disaccharidase levels in the jejunal mucosa by 10 hours after feeding. No appreciable change was noted in the six-hour biopsy in spite of the presence of ultrastructural abnormalities at that time. Although there is a turnover of disaccharidases within the surface epithelial cells (Rubenstein, Weser, and Sleisenger, 1966; Das and Gray, 1970) the half-life of the intestinal disaccharidases at least for the rat is 11·5 hours (James, Alpers, Gerber, and Isselbacher, 1971). If a similar turnover time is assumed for man, it is understandable that there was no change at six hours but an appreciable fall in enzyme levels by 10 hours.

The finding of morphological changes at six hours is important because the only other instance of changes having been noted so early followed the instillation of wheat into the ileum of two patients with treated coeliac disease (Rubin, Flick, MacDonald, and Parmentier, 1961). In the present study, the fraction B was fed by mouth and by 10 hours changes were well established, whereas Shiner (1973) found that feeding gluten by mouth resulted in changes only after about 11 hours and even then the changes were mainly ultrastructural.

The earliest evidence of mucosal damage observed by Shiner and her colleagues was hypertrophy of the endothelium of the capillaries and thickening of the subepithelial and capillary basement membranes (Shiner and Shmerling, 1972; Shiner, 1973). Although we observed similar changes, they were preceded by abnormalities in the surface epithelium. It is possible that these differences are related to the relative differences in toxicity between gluten and fraction B.

An inflammatory response similar to that seen in the present study has been demonstrated by Keusch, Grady, Takeuchi, and Sprinz (1972) after the introduction of Shigella enterotoxin into isolated ileal loops in the rabbit. These workers showed that six hours after the introduction of the enterotoxin there was shortening of the villi, degeneration and necrosis of epithelial cells, micro-ulceration, and increase in inflammatory cells in the lamina propria, the cells consisting mainly of lymphocytes which were infiltrating the epithelium in increased numbers.

One feature of our own results in favour of such a direct toxic action is the evidence of rapid mucosal recovery following damage by a single dose of fraction B. This suggests that only the epithelial cells in existence at the time of exposure to the fraction B are damaged and that the next generation reverts to normal, although additional studies are required to confirm this view.

Another possibility is that the fraction B is damaging through an immunological reaction. Shiner (1973) favours this view and suggests that the ultrastructural changes in the basement membranes and capillary endothelium are compatible with a complex mediated minimal response of the Arthus type.

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