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# Studies on Membrane Transport in Sensitized Rats. I. Absorption of Horseradish Peroxidase from the Intestine of Sensitized and Non-sensitized Rats

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It was demonstrated by electronmicroscopy and by the determination of enzymic activity in the mesenteric perfusate that horseradish peroxidase (HRP) was absorbed from adult rat intestine. When 1200 units of HRP was infused into the intestinal lumen of normal rats, 2.8 units of the enzyme was recovered in the mesenteric perfusate collected for 90 min.

On the other hand, the absorption of HRP decreased specifically in rats immunized with HRP. In an *in vitro* experiment using everted gut sac, the effect of intraperitoneal immunization (three times) on HRP influx was 2- to 3-fold greater than that of subcutaneous immunization (six times). Although the influx was unchanged in the rats injected subcutaneously three times, the recovery of HRP in the perfusate after the same sensitization decreased significantly compared with the control rats. These results suggest that the absorption of antigen decreases after active sensitization and that the effect of immunization on the intestinal absorption depends on the administration route of antigen.

**Keywords**——antigen absorption; immunization; horseradish peroxidase, intestine; mesenteric perfusion; electronmicroscopy; radioactive tracer

Recently, it has been established that only a little protein is absorbed from the intestine even after maturity. This finding evoked new interest in the relation between immunity and the absorption of an antigenic macromolecule such as a protein, namely, antibody production in response to an absorbed immunogen, antigen absorption in hypersensitivity, and so on. Isselbacher and his co-workers[1] have reported that the uptake of an antigen from the intestine specifically decreases after immunization. Their results were obtained by the *in vitro* everted sac method, and were suggestive of the interference of local antibody with antigen uptake. An experiment under more physiological conditions should provide useful information on the influence of systemic immunity on the intestinal absorption of antigen. Accordingly, an *in situ* mesenteric perfusion experiment was carried out in this study.

On the other hand, since the introduction of horseradish peroxidase (HRP) as a good tracer by Straus in 1957,[2] quite a few papers[3] have been presented on the uptake of HRP in various organs. The absorption of HRP through the small intestine in adult rat was histochemically demonstrated.[4] However, HRP transport into the portal vein has still not been investigated quantitatively.

The present paper deals with the results of an *in situ* mesenteric perfusion experiment, in which the absorption of HRP from the intestines of sensitized and non-sensitized rats was examined quantitatively.

## Materials and Methods

**Materials**——HRP (grade IV) and bovine serum albumin (frac. V) were purchased from Miles Lab.

Inc. *O*-Dianisidine (3,3'-dimethoxybenzidine dihydrochloride) and complete Freund's adjuvant (CFA) were products of Eastman Kodak Co., U.S.A. and of Iatron Laboratories, respectively. Histamine dichloride and diphenhydramine hydrochloride were obtained from E. Merck, Germany.

**Preparation of the Sample for Electronmicroscopic Observation**——The mesentery of a rat (about 180 g) was perfused with 0.1 M phosphate buffer, pH 7.4, and 5 mg of HRP was infused into the intestinal lumen. The test tissue was removed 3 or 10 min after HRP administration and prefixed with 2.5% glutaraldehyde in the foregoing buffer. The tissue was cut into small blocks 1 mm thick and rinsed with 8% sucrose for 18 h. The small blocks were immersed in Karnovsky's solution which did not contain any hydroxyperoxide and then were incubated in complete Karnovsky's solution (0.03% 3,3'-diaminobenzene/0.01% $H_2O_2$/0.1 M Tris HCl, pH 7.4) for 30 min.[5] The tissue blocks were rinsed with phosphate buffer several times, followed by post-fixation in 1% $OsO_4$[6] for 1.5 h. Subsequently, they were dehydrated with increasing concentrations of ethanol and embedded in Epon. Ultrathin sections were cut with a JEM-ultramicrotome and observation under a JEM-100 B electronmicroscope (JEOL Ltd.) was carried out directly or after staining of the sections with uranium acetate and lead citrate.[7]

**Immunization**——One mg of HRP dissolved in 0.25 ml of saline was emulsified with an equal volume of CFA. Female Wistar strain rats (6 weeks of age, about 100 g) were immunized by intraperitoneal or subcutaneous injections of 0.5 ml of the emulsion once a week. Control rats were injected with the same emulsion without any antigen. The animals were used in absorption studies one week after the last inoculation.

**Preparation of $^{125}$I-labelled HRP**——HRP (700 units/mg) was purified by gel filtration on Sephadex G-150 (Pharmacia, Sweden). According to Greenwood and Hunter,[8] 2 mg of the purified HRP (1700 units/mg) was labelled with 1 mCi of $^{125}$I (New England Nuclear) in the presence of Chloramine T.

**Determinaton of Peroxidase Activity**——Peroxidase activity was assayed according to the Worthington manual.[9] First, 1 ml of 0.3% (v/v) $H_2O_2$ was mixed with 0.83 ml of 1% (w/v) *O*-dianisidine in ethanol, and the volume was made up to 100 ml with 0.1 M phosphate buffer, pH 6.0. To 2.9 ml of the substrate solution kept at 25 °C, 0.1 ml of test solution was added. The reaction mixture was immediately poured into a cuvette, and the rate of color development at 460 nm was measured with a Hitachi spectrophotometer, model 124. One unit of peroxidase activity represents the amount of the enzyme decomposing 1 $\mu$mol of $H_2O_2$ per min at 25 °C.

The activity of the HRP preparations used was 700—1250 units/mg by this assay.

**Everted Sac Method**——According to Wilson and Wiseman,[10] 5 cm long everted sacs were prepared from the jejunum of rats, and about 0.5 ml of Krebs-Ringer bicarbonate solution, pH 7.4, was put into the sac. Two sacs were placed in 10 ml of the same solution containing 6000 units of HRP within a test tube and were incubated at 37 °C under constant gassing with 95% $O_2$-5% $CO_2$. After 30 min, the inner fluid was taken out and its volume and enzymic activity were determined.

***In Situ* Mesenteric Perfusion**——The method was described in our previous paper.[11] After preliminary perfusion for 30 min, various doses of HRP dissolved in 0.5 ml of saline solution were infused into the ligated jejunum of the rats (150—200 g). All sensitized rats were administered 1200 units of HRP. The perfusate from the mesenteric vein was collected for 90 min and the enzymic activity was determined every 30 min.

In the tracer experiment using $^{125}$I-labelled HRP ($3\times10^5$ cpm) radioactivity was measured with an Aloka gamma scintillation counter. An aliquot of the perfusate was dialyzed against deionized water (adjusted to pH 7.4 with NaOH) with stirring for 3 h to remove any free $^{125}$I.

**Schultz-Dale's Technique**——Rats which had been intraperitoneally immunized three times with the emulsion of HRP and CFA were sacrificed by exanguination after an overnight fast. The small intestine was immediately removed and flushed with Tyrode solution to exclude luminal debris. A segment of sensitized ileum was suspended in a 10 ml organ bath filled with aerated Tyrode solution at 37 °C and then 0.1 ml of HRP solution (4 mg/ml) was added to the bath. The contractile response of the ileum was recorded isometrically with a force-displacement transducer Model SB-TI and a multipurpose recorder Model RM-20 (Nihon Koden Kogyo Co.).

## Results

### Absorption in Normal Rats

Using an electronmicroscope we could observe HRP reaction products in the epithelial cells even 3 min after the administration. HRP-containing vesicles existed in the apical cytoplasm and in the supranucear region of the cells and their sizes were about 1 nm (Figs. 1, 2). The enzyme also appeared in the intercellular space between the absorptive cells within 10 min (Fig. 3). In the control preparation, the products were never found in the absorptive cells. Endogeneous peroxidase activity was sometimes noted in the lymphoid cells. However, this was easily distinguishable from exogeneous HRP reaction products. This histochemical investigation shows that HRP is rapidly absorbed through the rat intestinal epithelial cells after its administration.


Fig. 1. Electronmicrograph of Rat Intestinal Epithelial Cells 3 min after the Administration of HRP (×6000)

Many pinocytic vesicles can be seen in the apical cytoplasm of the cell; they contain the electron-dence reaction products of HRP (arrows). MV: microvilli, N: nucleus.

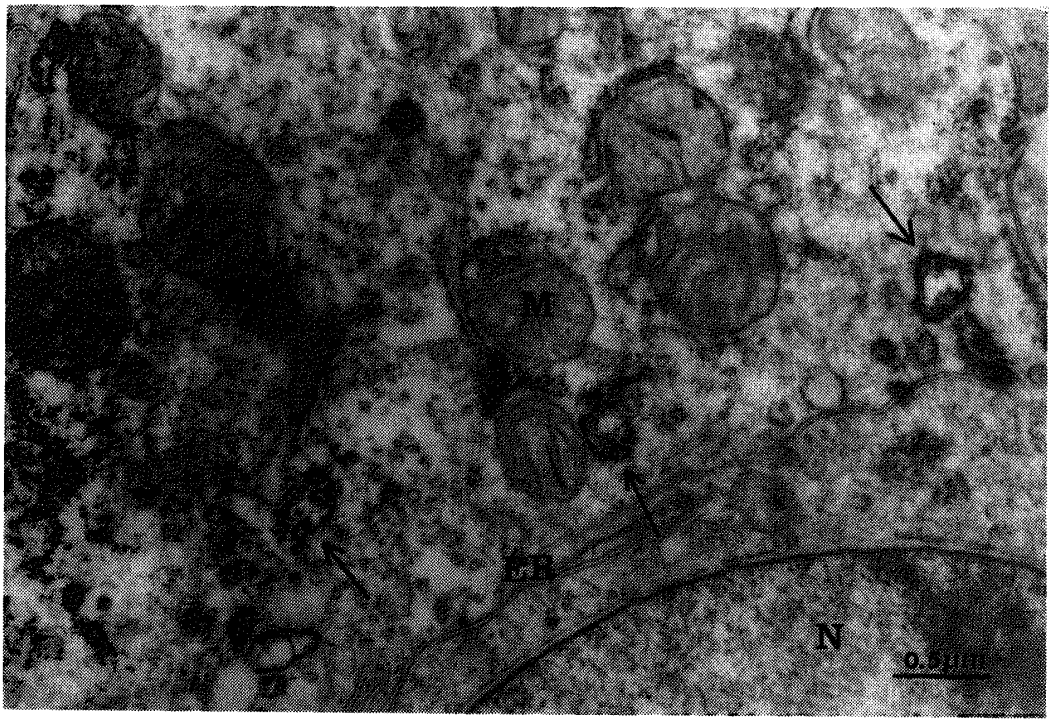


Fig. 2. Electronmicrograph of the Supranuclear Region of an HRP-containing Cell (×25000)

Small vesicles containing the reaction products of HRP can be seen (arrows). These vesicles seem to be transferred to the lateral region of the cell and to discharge HRP into the extracellular space. M: mitochondria, N: nucleus, ER: endoplasmic reticulum.

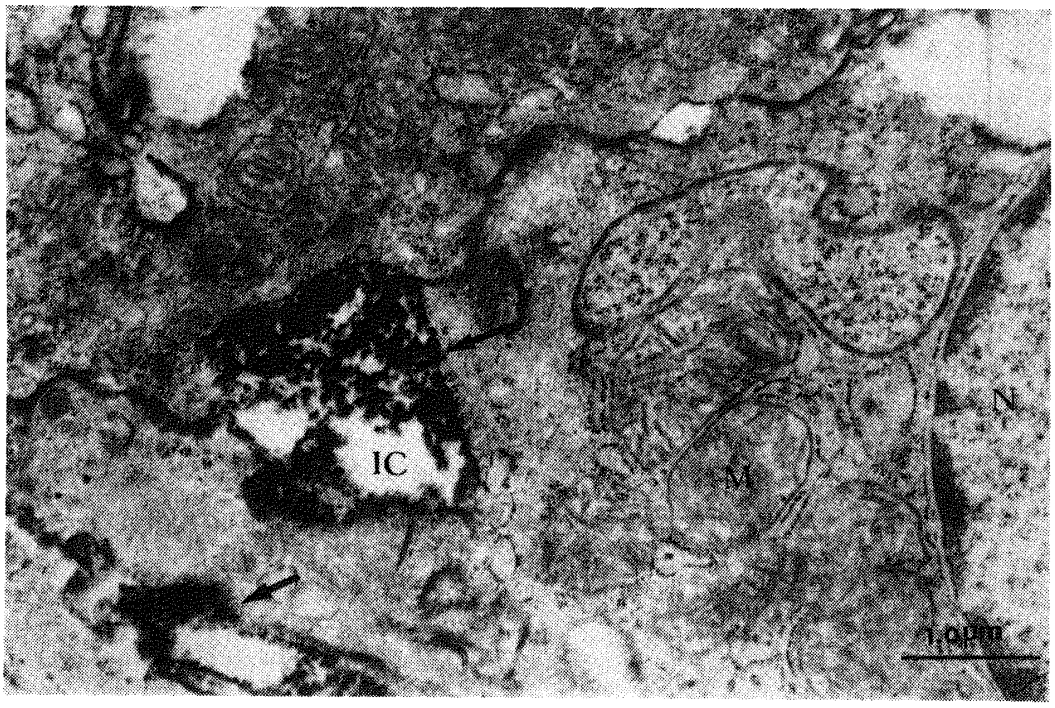


Fig. 3. Electronmicrograph of Lateral Region of an HRP-containing Cell (×20000)
M: mitochondria, N: nucleus, IC: intercellular space.

In the *in situ* experiment, some rats were administered 2.5 mg of bovine serum albumin, but no peroxidase activity was detected in the perfusates collected after and before sample administration. However, shortly after the infusion of HRP into the lumen the activity appeared in the perfusate. The perfusate (0—30 min), the HRP employed and the mixture of two were subjected to gel filtratoin on Sephadex G-150. Elution profiles of peroxidase activity of these samples are shown in Fig. 4; they all coincide. These results indicate that the active component in the perfusate is derived from the administered HRP.

Table I shows the recoveries of radioactivity and enzymic activity in the perfusate specimens collected after the ingestion of 525 units of HRP containing $^{125}$I-labelled enzyme. HRP recovered in 90 min amounted to 1.43 units, equivalent to 0.27% of the administered amount. Subsequently, three perfusate specimens collected every 30 min for 90 min were dialyzed individually. About 49, 29 and 22% of the radioactivities recovered in the respective specimens were found to be still bound to macromolecules. This means that about 0.3% of $^{125}$I-HRP was recovered as $^{125}$I-bound macromolecules in 90 min. This recovery agreed well with that of HRP activity above mentioned. The recovery of HRP increased with increasing dose in the intestine up to about 2000 units (Fig. 5).

**Absorption in Sensitized Rats**

Schultz-Dale's technique is a useful tool to demonstrate anaphylactic hypersensitivity.[12] The ileum of HRP sensitized rat showed a typical Schultz-Dale contraction when the challenge was given one week after the last immunization (Fig. 6). Moreover, the sera from immunized rats were incubated at 56°C for 30 min, and antibody titers were determined by means of the ring test. The serum of rats injected with HRP three times either by the subcutaneous route or by the intraperitoneal one formed a precipitin ring against HRP of 0.005% upward. The rats injected subcutaneously six times gave higher antibody titers of 0.001—0.0025%.

HRP influx from the everted guts of hypersensitive rats is shown in Fig. 7. Control rats and rat immunized with an unrelated antigen, semi-alkaline proteinase, showed no difference in influx compared with the intact group. Similarly, there was no significant difference

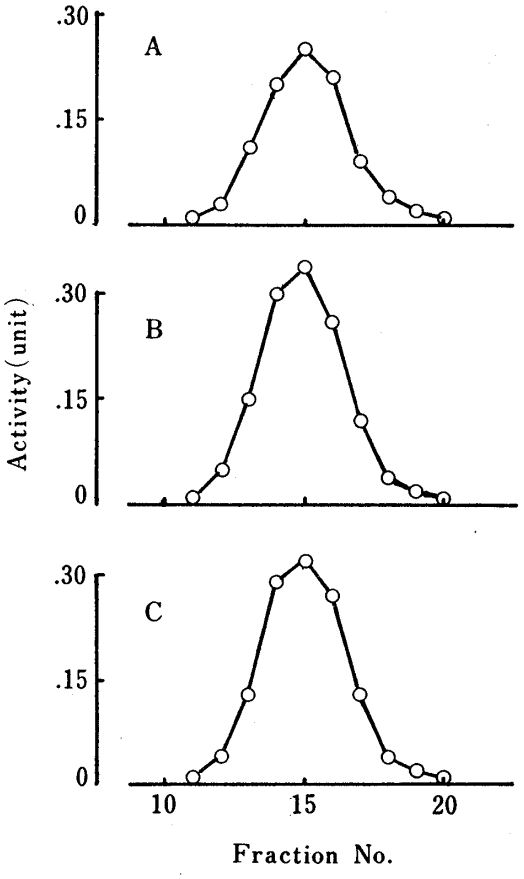


Fig 4. Elution Profiles of Peroxidase Activity in Gel Filtration of the Mesenteric Perfusate and Administered HRP

About 2 ml of sample solution was applied to a Sephadex G-150 column (1.5×45 cm). Elution was carried out with 0.1 M phosphate buffer (pH 6.0) at 4°C and fractions of 3.3 ml were collected. A: HRP administered, B:perfusate (0—30 min), C: mixture of HRP and perfusate.

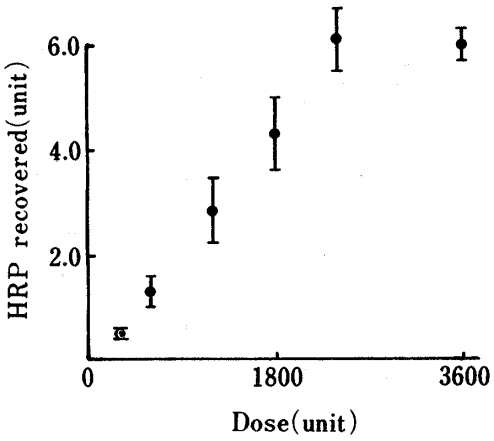


Fig 5. Peroxidase Activity recovered in Mesenteric Perfusates after Administered of Various Amounts of HRP

between the influxes of rats immunized subcutaneously three times with HRP and control rats. However, after the sixth injection, HRP transport remarkably decreased as shown in Fig. 7. Lower transport was found in rats sensitized intraperitoneally three times with HRP and amounted to only about 13% of that in intact rats.

In the *in situ* experiment, suppression of HRP absorption was also recognized in the control rats. When 1200 units of HRP was administered into the intestinal lumen of intact rats, 2.8 units of HRP was recovered in 90 min. The recoveries in control groups were 1.51 and 0.36 units in the cases of subcutaneous and intraperitoneal injections, respectively. Rats sensitized with the emulsion containing bovine serum albumin gave the same result (control group). However, the reduction of the absorption was larger in HRP sensitized rats than in the control. In the animals sensitized three times with subcutaneous injection of HRP, the HRP recovery in the perfusate decreased to 20% of that in intact rats and to 38% of the control (Table II). Such suppression was not found in the everted gut from the same group.

TABLE I. Recoveries of Peroxidase Activity (I) and $^{125}$I-Bound Macromolecules (II) in Mesenteric Perfusate

| | % of administered activity (mean±S. E.) | | | |
|---|---|---|---|---|
| | 0—30 | 30—60 | 60—90 | 0—90 min |
| I | 0.20±0.04 | 0.05±0.02 | 0.02±0.01 | 0.27±0.06 |
| II | 0.19±0.03 | 0.07±0.02 | 0.04±0.01 | 0.30±0.09 |
| | (0.39±0.07) | (0.24±0.06) | (0.18±0.04) | (0.81±0.16) |

Rats were given 525 units of HRP in the intestinal lumen.
Recovery of total radioactivity is shown in parentheses.

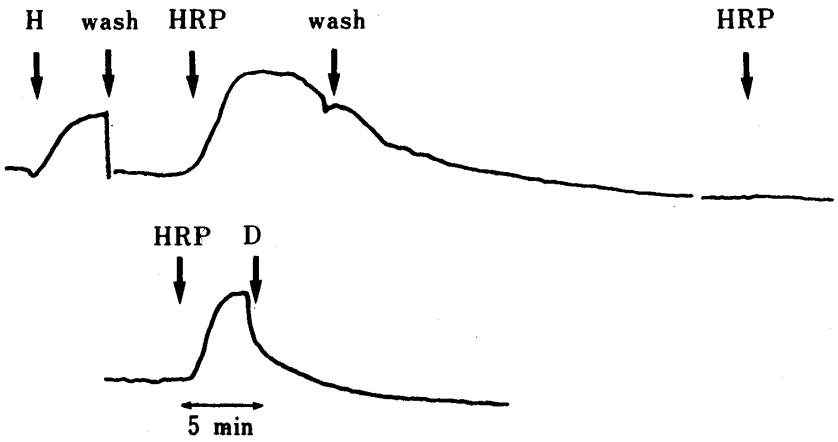


Fig 6. Anaphylactic Contractions of the Rat Ileum Sensitized with HRP
H: histamine (1 mg/ml, 0.1 ml), D: diphenhydramine (1 mg/ml, 0.1 ml).

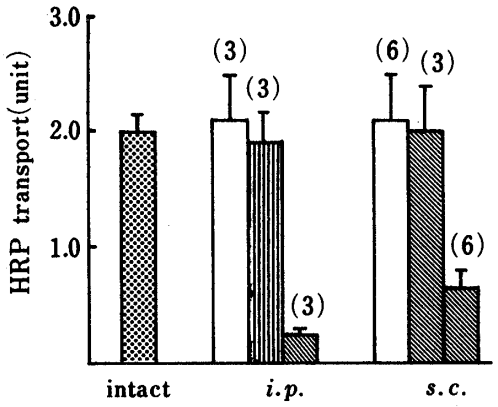


Fig. 7. Effect of Parenteral Immunization on HRP Transport across the Everted Intestine
The number of times of ingection is represnted in parentheses.
▦ : intact, □ : control, ▥ : immunized with semialkaline proteinase, ▨ : immunized with HRP.

## Discussion

It has been demonstrated by electronmicroscopy that horseradish peroxidase (molecular weight, 40000) is absorbed across the adult rat intestinal membrane. The enzyme seems to be absorbed immediately after administration into the lumen. After 3 and 10 min, it was observed in the cytoplasm and intercellular space, respectively. These findings are consistent with the quantitative results of enzyme activity in thc mesenteric perfusate, which showed that the recovery was highest up to 30 min after the administration (Table I). The recovery at 90 min was about 0.2% of the administered amount, and above a certain dose (about 2500 units) the amount absorbed became almost constant.

The intraperitoneal injection of CFA produced inflammation and adhesion of the rat intestine. However, the decrease of HRP absorption in HRP immunized rats was not due to the injury to the tissue, because it was not observed in the removed intestine of control rats injected CFA emulsion without any antigen. Neverthless, the recovery of HRP in the *in situ* mesenteric perfusion experiment was decreased non-specifically by the injection of CFA emulsion alone, especially by the intraperitoneal route. The findings are very interesting, but can not be explained at present.

TABLE II. Effect of Subcutaneous Immunization (Three Times) on the Recovery of HRP in Mesenteric Perfusate

| Rat | HRP recovered (unit) | % of intact |
|---|---|---|
| Intact | 2.84±0.59 | 100 |
| Control | 1.51±0.25 | 53 |
| BSA immunized[a)] | 1.61±0.35 | 57 |
| HRP immunized | 0.58±0.13 | 20 |

*a)* Rats were immunized with bovine serum albumin.

The absorption of HRP decreased markedly in HRP-immunized rats compared with intact and control rats, but not in rats immunized with an unrelated antigen. These findings indicate that specific antibody is involved in the interference with the intestinal absorption of the antigen. The suppression rate of HRP influx from the removed gut was higher in rats immunized by the intraperitoneal route than by the subcutaneous route (Fig. 7). From these results of the *in vitro* experiment, it is conceivable that antibody localized in the intestine is the cause of the suppression, and is induced more easily by local stimulation with the antigen. Subcutaneous immunization (three times) had no effect on the influx from the everted gut. However, the absorption of antigen in the *in situ* experiment was remarkably decreased in comparison with the control. The contrary results in the two experiments suggest a specific impairment of antigen absorption even in the absence of local antibody. The antibody titers of these immunized rats were high, and thus interference of systemic immunity with antigen absorption probably occurs. Further work is in progress.

There are recent papers showing that the specific antibody is present in the plasma of an animal orally given egg albumin[13] and that parenteral challenge following previous ingestion of immunogenic protein induces a specific hyporesponsiveness.[14] The intestinal absorption of immunogenic macromolecules has also been studied from the viewpoint of alimentary allergy. Though the amount of macromolecular protein absorbed in a mature animal is extremely small in quantity,[11,15] it may be significant in relation to the immunoresponse and immunoreaction.

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