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EXTRACTION OF GASTRIC PARIETAL CELL AUTOANTIGEN

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SUMMARY

The distribution of gastric parietal cell autoantigen in subcellular fractions of bovine abomasum mucosa has been assessed by the capacity to neutralize anti-gastric parietal cell activity of pernicious anaemia serum. By far the greater proportion of the antigenic activity was in the microsomal fraction. Active material was soluble in 6 M-urea, pH 7·3. Removal of urea by dialysis produced an active suspension which on centrifuging gave an active sediment and an inactive supernatant. Electronmicroscopic and ultraviolet absorption evidence suggests that the antigenic component(s) of the microsomal fraction is associated with the smooth membranes and not with the ribosomes.

INTRODUCTION

Pernicious anaemia patients show a high incidence of circulating antibodies which react with the gastric parietal cell (Taylor *et al.*, 1962; Irvine, 1963; de Boer, Nairn & Maxwell, 1965) and some attempts have been made to characterize the antigenic component(s) concerned (Baur, Roitt & Doniach, 1965). One of the problems associated with this work has been the difficulty of bringing the antigen(s) into solution, hindering analysis by conventional biochemical and immunochemical methods.

This paper describes the extraction of gastric parietal cell antigen from gastric (abomasum) mucosa of cattle and its solution by 6 M-aqueous urea.

METHODS

Tissue fractionation

The abomasum mucosa of cattle, used as the starting material, was selected as the most suitable after examination of the stomachs of several other species. The use of human,

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monkey or rat stomach was rejected because of inadequate supply of mucosa, and pig and sheep stomachs appeared to be poorer in antigen. The abomasum, obtained at the abattoir within 15 min of the death of the animal, was rinsed several times in physiological saline and transported to the laboratory in a plastic bag packed in ice. Within 2 hr of collecting the tissue, after further washing in ice-cold saline, the mucosa and sub-mucosa were dissected away from the muscle and washed with the 'homogenizing' buffer solution which had the following composition: 0·25 M-sucrose, 0·01 M-KCl, 0·005 M-$MgCl_2$, 0·02 M-2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), with HCl added to pH 7·3. The washed tissue was either diced by hand or cut into strips and passed through a plastic domestic meat mincer fitted with metallic cutters and shaft ('Moulinex'). These and all subsequent tissue fractionation and extraction operations were performed at 0–4°C in a cold room, refrigerated centrifuge, or with apparatus immersed in ice-water. The mucosa, in 30% (w/v) suspension in the homogenizing buffer, was disintegrated in a Silverson Model B homogenizer using a coarse cutter and then a fine cutter for approximately 3 min in each case. Sometimes the mucosal tissue was stored for up to 3 months at −30° or −70°C before homogenization, for which the frozen material was thawed at 37°C until sufficiently soft to slice with a knife for mincing. No appreciable loss of antigenic activity was detected with such storage. Any storage of subsequent fractions was carried out at −30°C.

For subcellular fractionation the homogenate was centrifuged at 500 ***g***, calculated for maximum radius, for 15 min in 50 ml cellulose nitrate tubes in a Servall RC-2 centrifuge to remove coarse fragments and nuclei. The supernatant was decanted and re-centrifuged at 10,000 ***g*** for 30 min to remove the mitochondrial fraction. The new supernatant was siphoned off and centrifuged at 105,000 ***g*** for 90 min in the type 30 rotor of a Spinco L-2 ultracentrifuge: the supernatant cell sap was retained and the microsomal sediment was washed by resuspension in the homogenization buffer and recentrifuged. The tubes were drained and the sediment was washed twice more in the same way.

Chemical procedures

Reagents. Double-glass-distilled water was used. Phosphate-buffered saline was 0·145 M-NaCl, 0·01 M-phosphate, pH 7·1. Tris was 'Sigma 7-9' (Sigma Chemical Co.). Guanidine hydrochloride was 'Biochemical' grade (British Drug Houses). All other chemicals were A.R. grade.

Dry weight was assessed on water-washed sediments brought to constant weight by heating at 110°C and cooling over anhydrous magnesium perchlorate for 20 min.

Nitrogen content was determined by a micro-Kjeldahl technique.

Ultraviolet absorption measurements were made in 1 cm cells in a Unicam SP500 spectrophotometer. Preparations were diluted in 0·145 M-NaCl, 0·01 M-phosphate, pH 7·0, to obtain extinctions (E) between 0·20 and 0·45. In addition to the frequently used relationship of $E_{260m\mu}$: $E_{280m\mu}$ as an approximate measure of the ratio of nucleic acid to protein, $E_{260m\mu}$: $E_{235m\mu}$ was also employed, as recommended by Petermann (1964a).

Urea extraction. The washed microsomal sediment was suspended in the homogenization buffer to give a volume which was 5% of that of the first microsomal fraction, i.e. of the 10,000 ***g*** supernatant. Urea (8 M) in 0·01 M-KCl, 0·005 M-$MgCl_2$, 0·02 M-Tris and HCl to pH 7·3, was added to the sediment suspension in a slow stream with constant mixing until

the final volume was four times the original. Cooling of the 8 M-urea solution before the addition sometimes resulted in crystallization which was reversed by warming just sufficiently to re-dissolve the urea. The effect of the urea solution on the sediment suspension was to produce an immediate substantial clearing. The resultant mixture, with a urea concentration now 6 M, was centrifuged at 105,000 ***g*** in the Spinco type SW 39L rotor for 90 min and the supernatant pipetted off; a slightly turbid surface layer was removed separately. The sediment was sometimes further extracted with 6 M-urea in the KCl–$MgCl_2$–Tris buffer.

Guanidine hydrochloride extraction. This was carried out in the same manner as the urea extraction. Guanidine hydrochloride (7 M) in the KCl–$MgCl_2$–Tris buffer was used, and its final concentration in the mixture was 5 M.

Immunological assay

Serological absorptions for testing antigenicity were carried out with the subcellular fractions and the 6 M-urea extract: this solution was first dialysed for 24 hr against three changes of phosphate-buffered saline to yield a suspension (DUS). The dialysis was adopted to avoid possible complicating effects of urea on subsequent serology though preliminary testing indicated that the urea extract had much the same antigenic activity as its dialysed product. Tests were also carried out on the 105,000 ***g*** centrifuged sediment and supernatant from this suspension. All volumes were adjusted with phosphate-buffered saline to that of the original microsomal fraction. For some tests more concentrated samples were also examined.

Antigenic potency of the various products was assessed from their capacity to neutralize the anti-gastric parietal cell activity of pernicious anaemia serum. Immunofluorescent staining of rat stomach sections was employed as the test system (de Boer *et al.*, 1965). For such testing a few pernicious anaemia sera were selected which gave brilliant staining (+ + +) of gastric parietal cells, using the sandwich method with goat anti-human-globulin conjugated with lissamine–rhodamine B (RB 200) (Nairn, 1964). One drop of test preparation and one drop of pernicious anaemia serum were mixed and incubated at 37°C for 1 hr and centrifuged at 5,000 ***g*** for 5 min: the supernatant was then applied to a rat stomach section as the first layer in the sandwich staining procedure.

Electronmicroscopy

Preparations, fixed for 1½ hr in 1% osmium tetroxide in 0·075 M-sodium phosphate, pH 7·3, were dehydrated in acetone and embedded in Araldite for sectioning. Uranyl acetate and lead citrate were used for staining and the sections were examined with a Siemens Elmiskop 1 microscope.

RESULTS

Table 1 provides a comparison of antigenic activity of the subcellular fractions and extracts, as measured by their ability to neutralize gastric parietal cell autoantibody. By far the greatest activity was found in the microsomal fraction and the small amount detected in the nuclear, mitochondrial and cell sap fractions might be attributed to microsomal contamination. After extraction of the microsomal fraction by the 6 M-urea solution, the residue obtained

by centrifuging at 105,000 ***g*** was significantly less active than the DUS obtained by dialysing the supernatant against phosphate-buffered saline. The activity was located in the 105,000 ***g*** centrifuged sediment of the DUS, not in the supernatant. It is clear that the antigenic material is largely extracted by the urea solution, and is reprecipitable by dialysis against phosphate-buffered saline. The 6 M-urea solution gave optimal extraction of antigen: more dilute solutions left a larger undissolved residue with antigenic activity and more concentrated solutions crystallized in the cold.

TABLE 1. Effect of absorbing pernicious anaemia serum with various subcellular fractions on the immunofluorescent staining of gastric parietal cells

Serum absorptions	Nil	Nuclear, mitochondrial and cell sap fractions	Original microsome fraction	Residue from urea extraction	Dialysed urea extract suspension (DUS)	Centrifuged DUS	
						Sediment	Supernatant
Immunofluorescent staining	+++	++	(−)	+	(−)	(−)	+++

(−) = Substantial inhibition of staining usually complete.

There was no improvement in specific activity of the preparations as assessed by dry weight and nitrogen comparisons of antigenically equivalent microsomal fractions and DUS sediments, but 60% of the DUS nitrogen was located in the inactive centrifuged supernatant. The estimated proportions of protein and RNA in the DUS and its centrifuged products (Table 2) indicate that nitrogenous material, rich in RNA or its derivatives, has

TABLE 2. Proportions of protein and RNA in dialysed urea extract suspension (DUS) and fractions

Fraction	Proportion of total nitrogen	Nominal RNA:protein	
		$\frac{E_{260m\mu}}{E_{235m\mu}}$	$\frac{E_{260m\mu}}{E_{280m\mu}}$
DUS	1·0	0·92	1·52
Sediment	0·4	0·73	1·19
Supernatant	0·6	1·47	1·93

been removed in the inactive centrifuged DUS supernatant. These findings indicate a degree of chemical purification accompanied by immunological denaturation.

The 5 M-guanidine hydrochloride extraction was effective but gave somewhat poorer results than the urea extraction and was therefore abandoned.

Electronmicroscopy of the antigenically potent microsomal fraction of bovine abomasum mucosa confirmed the presence of smooth membrane vesicles, ribosomes and occasional rough membrane vesicles (Fig. 1a). In the residue from the urea extraction

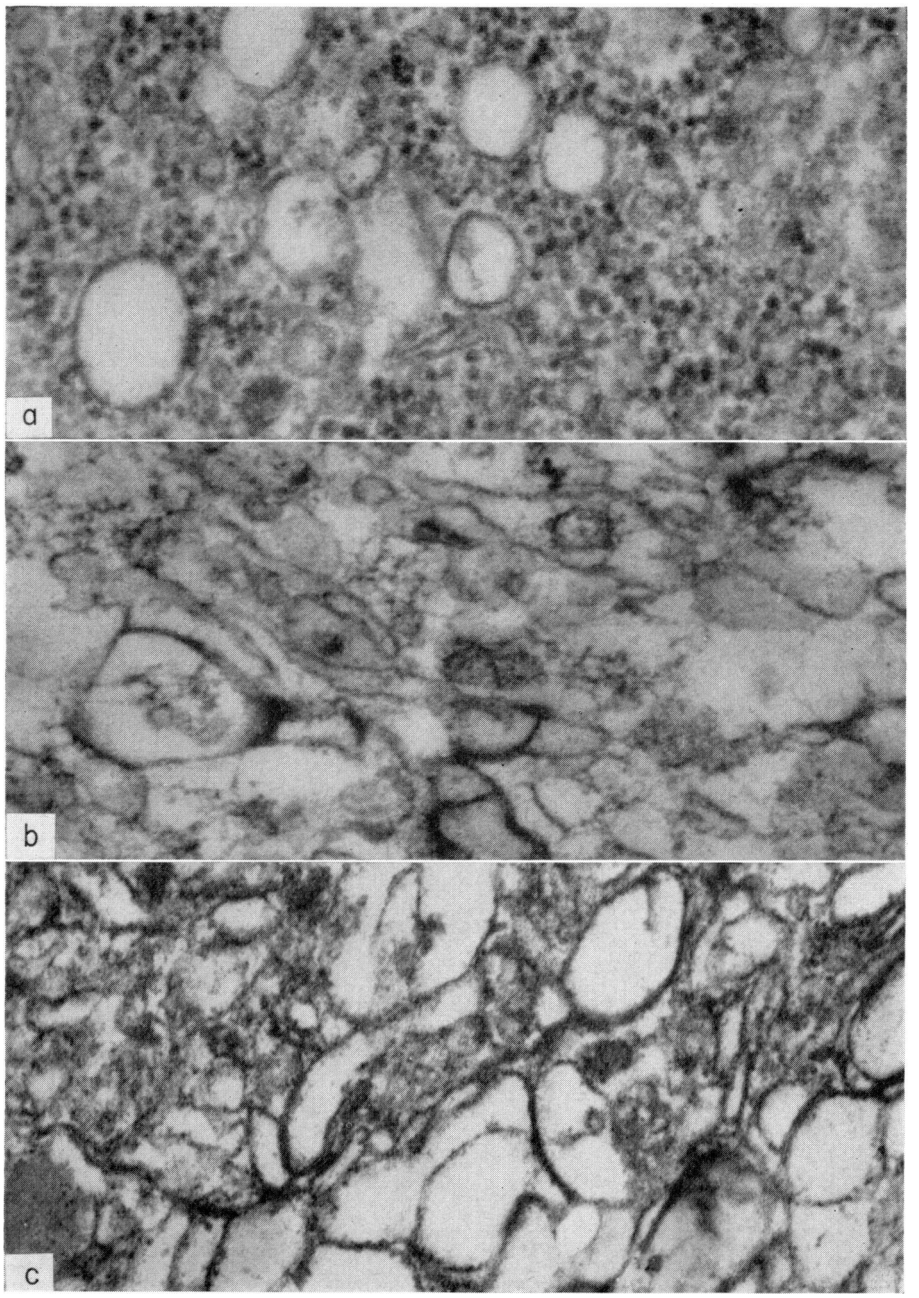

FIG. 1. Electronmicrographs of fractions of bovine abomasum mucosa, × 90,000. (a) Microsomal fraction showing ribosomes, smooth membrane vesicles and, bottom left, one rough membrane vesicle. (b) Residue after urea extraction of microsomal fraction, showing smooth membrane vesicles only. (c) Sediment from dialysed urea extract of microsomal fraction, showing smooth membrane vesicles only.

(Fig. 1b) and the DUS sediment (Fig. 1c) only smooth vesicles are recognized, the ribosomes apparently having been disrupted during the extraction procedure.

DISCUSSION

The ultraviolet absorption data and nitrogen estimations show that the final antigenically active DUS sediment is poor in RNA in comparison with the original DUS and the inactive supernatant. Moreover the electronmicroscopical findings show that the activity is unrelated to the presence of rough membranes or ribosomes; the latter are presumably disrupted in 6 M-urea solution (see Petermann, 1964b). These observations favour the view that the gastric parietal cell autoantigen is a component of the smooth membranes, which is in accord with the conclusions of Baur *et al.* (1965). It is possible that the structures which are visible in the electron-microscope are not themselves responsible for the antigenic activity which could be due to a component, for example a product of ribosome breakdown, adsorbed on the vesicles, but we have no evidence of this.

Urea and guanidine hydrochloride, the two reagents reported here to bring about solution of the gastric antigen, possibly operate by virtue of their hydrogen bond-breaking properties. Disruption of hydrophobic and electrostatic bonding with consequent molecular unfolding may also have exerted an effect (Nozaki & Tanford, 1963; Katz & Ferris, 1966). The antigenic integrity of the urea extract has been established by its capacity to neutralize the anti-gastric parietal cell activity of pernicious anaemia serum. Furthermore the extract has been used as an immunizing antigen in rabbits to produce an antiserum with specific activity against the gastric parietal cell (Ward, de Boer & Nairn, to be published). No definite statement can be made about the chemical nature of the antigen concerned from these preliminary studies but its solution in 6 M-urea while still retaining antigenic activity may prove a promising starting point for further fractionation and characterization.

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