more. Perhaps as a result of this increase in sensitivity, nonspecific reactions were weaker. Washing in buffer for 12–18 h at 4 °C also diminished nonspecific reactions. Initially various 'glues', including albumen, gelatin and araldite, were used to prevent the sections floating off the slides during the period of treatment with trypsin but the short incubation times in the enzyme solution made their use unnecessary. When enzyme treatment was optimal, the excellent preservation of the histological structure was unaffected and the sections reacted to high titres with the PAP sequence with very little non-specific staining. The results obtained with trypsin-treated tissues fixed in glutaraldehyde/formalin/calcium acetate solution were particularly impressive.

Discussion. In immunohistochemistry, prior fixation helps to stop antigens diffusing away from their sites within the tissues and by preserving the structural integrity of the tissue facilitates detailed histological study. Nevertheless it is well-known that fixation often renders a tissue immunologically unreactive and it tends to be assumed that the fixative has either destroyed the antigen or allowed it to diffuse away. The ease with which trypsin unmasks antigen suggests that when carefully-fixed tissue fails to react immunologically, the cause is more likely to be fixative-induced impermeability of the tissue rather than actual loss or destruction of the antigen by the fixative. Trypsin presumably acts by causing some disruption of tissue structure but this appears to be at the ultrastructural or molecular level since it is not apparent in the light microscope in sections optimally exposed to the enzyme. It is difficult to postulate a specific site of action for trypsin, particularly in view of the fact that other experiments have shown that sialidase (100 U ml^{-1} at pH 5.5) and streptokinase (100 IU ml^{-1} at pH 7.8) can be used instead of trypsin but with rather less effect. Whatever the action of the enzymes on the tissues, it is undoubtedly facilitated by the fact that the reactions in the PAP sequence take place on the surface of the tissue section, as can be shown by cutting a section of a section which has been treated in this way (unpublished observations). The enzyme would therefore only have to 'etch' the surface of the section of tissue in order to influence its responsiveness, and the short incubation times tend to confirm this. The other experiments also showed that trypsin can unmask antigens other than Ig, including carcino-embryonic antigen in carcinomas of breast and colon and the α subunit of human chorionic gonadotrophin in breast carcinoma. The results confirm that trypsin treatment is so efficient at unmasking antigens in tissue sections that it should be employed routinely when fixed tissues are used in immunohistochemical procedures.

A simple enzymatic method for isolation of hepatocytes

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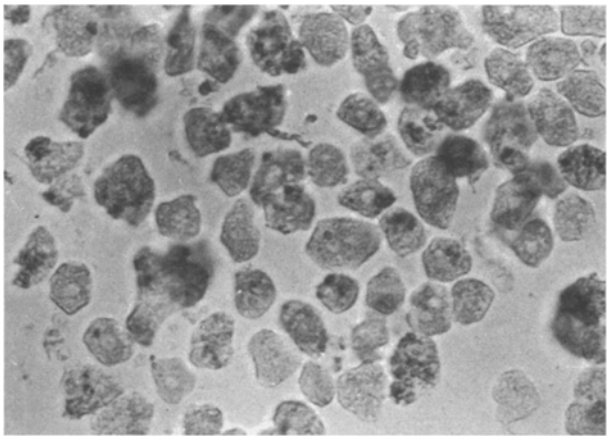
Summary. A new method is described for isolating hepatocytes by an enzymatic procedure: 0.05% bacterial collagenase in calcium-free Hanks' solution is injected into the liver tissue which is removed aseptically. The time required to prepare culture and risk of bacterial and fungal contamination is greatly reduced.

A new technique for the isolation of hepatocytes was developed. For the isolation of hepatocytes, enzymatic digestion with trypsin, bacterial collagenase or hyaluronidase has commonly been used[1] and recently in order to obtain high yield of cells, various perfusion techniques have been employed[2–4]. However, these perfusion techniques require a considerable degree of skill and take a long time. Furthermore, because of the long time required for the procedures, cultures are exposed to increased risk of bacterial and fungal contamination. Our new technique is very simple and the time required is reduced to $^1/_2$ to $^1/_3$ compared to that required by hepatic perfusion methods of enzymatic solution.



Isolated hepatocytes suspended in Eagle's minimum essential medium containing 20% of fetal calf serum.

Materials and method. Liver was removed aseptically as a whole from rats of Wistar strain, weighing 100–150 g, and washed 2–3 times with physiological saline; 0.05% bacterial collagenase (cl. histolyticum, purity grade 11, Boehringer, Mannheim) in calcium-free Hanks' solution, warmed to 37 °C in advance, was injected into the liver tissue at the speed of 10 ml/min through a tuberculin needle equipped to a sterile syringe. With infiltration of the enzyme solution into the tissue, a diffuse whitish colour change of the whole liver was observed. If the colour change was only local around the injected site, the enzyme solution was injected at several different sites. After the injection of about 20 ml of collagenase solution, the liver was washed once in calcium-free Hanks' solution and then torn into small pieces with 2 forceps in a Petri dish containing 10–20 ml of calcium-free Hanks' solution. Hepatocytes were liberated during this procedure. Cal-

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cium-free Hanks' solution containing isolated hepatocytes and torn tissue was filtered through a platinum sieve with 150 meshes. The filtrate was centrifuged at a very low speed (200 rpm) for 3 min. The supernatant was decanted. The residue was resuspended in about 10 ml of Eagle's minimum essential medium (MEM) and centrifuged again at low speed. The supernatant was decanted. The same procedures were repeated. The red blood cells were removed by this procedure and the isolated hepatocytes were used for primary culture. The cell viability was judged by the following 3 criteria: a) exclusion of trypan blue, b) the ability to synthesize glucose from alanine or lactate and c) the uptake of radioactive amino acid into the newly synthesized protein. Glucogenic ability was measured by adding appropriate amounts of alanine or lactate (2 μmoles/dish) to 4 ml of fetal-calf-serum-free MEM containing 5×10^6 cells. Glucose content was assayed using glucose oxidase. The protein biosynthesis was judged by incorporation of L-^{14}C-leucine (348 mCi/mmole, The Radiochemical Centre, Amersham). 2 μCi of ^{14}C-leucine was added to 4 ml of culture media (MEM) containing 5×10^6 cells and incubated for 3 h. Cells were washed several times with 1% cold trichloracetic acid solution and then appropriate amounts were dissolved in 1.0 ml of 0.1 N NaOH, mixed with Bray's solution and activity of radioactive carbon was determined by Packard Tricarb 3115 scintillation counter.

Glucogenic capacity from alanine or lactate and amino acid incorporation into the newly synthesized protein in the isolated hepatocytes

	Trypan blue staining	Glucose production (nmoles/h/10^6 cells) Alanine	Lactate	Incorporation of ^{14}C-leucine (pmoles/min/10^6 cells)
Exp. 1	5% >	7.67	16.33	2.12
Exp. 2	5% >	8.67	10.87	3.95

Values are means from duplicated samples.

Results and discussion. Several washings removed red blood cells in the hepatocyte suspension almost completely. The recovery of hepatocytes after centrifugations was more than 90% of the amount of the initially isolated cells, and the average number of the isolated hepatocytes obtained from 10 experiments was $(7.86 \pm 4.24) \times 10^6$/g wet liver tissue. As shown in the figure, the isolated cells were a mixture of polygonal and round ones, and it was demonstrated by Roser's albumin floatation method[5] that the contamination of mesenchymal cells, especially Kupffer cells was less than 1%. More than 95% of the isolated hepatocytes excluded trypan blue. As shown in the table, the rate of glucose production from 2 μmoles of alanine or lactate added to the culture media was 7.76 and 8.67 nmoles/h/10^6 cells and 16.33 and 10.87 nmoles/h/10^6 cells, respectively. The amount of ^{14}C-leucine incorporated into the newly synthesized protein was 2.12 and 3.95 pmoles/min/10^6 cells in 2 separated experiments.

The new method for isolating hepatocytes described above proved to be excellent, because it can be performed very easily in a short time and because of lower risk of bacterial and fungal contamination during isolation procedure.

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Large-scale purification of inactivated influenza vaccine using membrane molecular filtration

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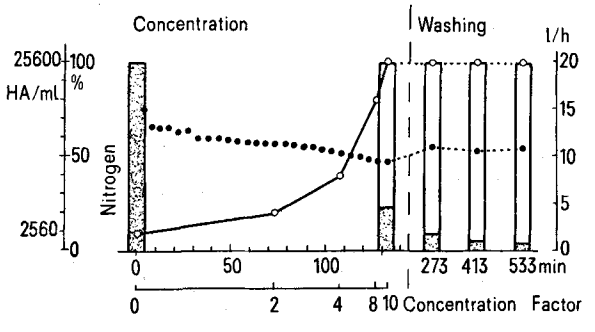
Summary. A procedure is reported for the elimination of at least 95% of hens' egg protein impurities from inactivated influenza vaccine, by selective molecular filtration through a membrane with a cut-off limit of 1×10^6 daltons.

The current problem in the production of inactivated influenza vaccine is the complete elimination of hens' egg protein impurities present in the allantoic fluid, in order to minimize the high incidence of post-vaccinal reactions. Of the several methods which have been published for large scale purification of inactivated influenza virus suspensions[1–6], the zonal ultracentrifugation technique is the most widely used[3,4]. As these methods have some drawbacks, other physical techniques can offer a more advantageous approach to facilitate elimination of soluble impurities from influenza virus suspensions.

Membrane ultrafiltration has been used until now only as a concentration step. In this paper we have reported the results obtained using the membrane with cut-off limits of 1×10^6 daltons, in the large-scale purification of influenza vaccine.

Results and discussion. Purification of influenza vaccine is based on the fact that most impurities are soluble macromolecules with a mol. wt less than 1×10^6 daltons,



Concentration and purification of 30 l of inactivated influenza A (MRC 12) virus suspension. The columns represent the ratio of PN in the retentate (black) and filtrate (white). Flow rate (●) and HA activity (○) are also given. The purification is represented by an analysis of 3 washing volumes.

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