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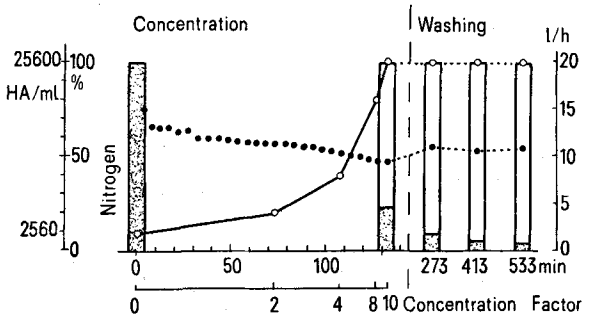
Summary. A procedure is reported for the elimination of at least 95% of hens' egg protein impurities from inactivated influenza vaccine, by selective molecular filtration through a membrane with a cut-off limit of 1×10^6 daltons.

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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Method	Virus	Volume (ml) Start	Volume (ml) Final[a]	PN (mg/ml) Start	PN (mg/ml) Final	PN eliminated (%)	HA/mg PN Start	HA/mg PN Final	Purification factor y	Virus recovery[b] (%)
MF	B×1	4100	695	0.776	0.024	96.9	827	25725	31.1	100
MF	A-MRC 12	30000	3000	0.644	0.027	95.8	3975	94815	23.8	100
MF	B×1	30000	3000	0.721	0.029	96.0	718	17655	24.6	100
MF + chromatography	B×1[d]	50	83	0.11	0.026	76.4	72727	280827	3.9	93.3
MF + chromatography	B×1[e]	50	265	0.938	0.600	36	68230	88274	1.3	82.8
MF + zonal ultracentrifuge	A-MRC 12[d]	3000	100	0.27	0.032	88.1	94815	686185	7.2	86.7

[a] PN recalculated according to the initial volume; [b] Virus recovery expressed as HA value. HA tests performed at room temperature in tubes using 2fold dilutions of virus in 0.5 ml of PBS + 0.5 ml RBC. HA was read as the highest dilution causing complete RBC agglutination; [c] Sepharose 2B chromatography performed on 3,7×54 cm column; [d] Virus concentrated 10×; [e] Virus concentrated 100×.

and which could therefore be separated from virus particles by membrane molecular filtration. The process has been performed with the Pellicon® Cassette System, using PSVP membranes with a cut-off limit of 1×10^6 daltons (Millipore, Bedford, Mass., USA), and a piston pump generating 4 at working pressure.

For these experiments, membranes with a total surface of 4600 cm^2 were chosen. Lots of MRC 12 type A and B×1 type B inactivated influenza virus strains were used in amounts of 4–30 l. The starting suspensions had a protein nitrogen content (PN) of 0.6–0.8 mg/ml; hemoagglutinin value (HA/mg PN) 1100–4000 for type A and 700–2000 for type B. Positive polyacrylamide gel electrophoresis (PAGE) and immunoelectrophoresis (IEP) against hens' egg proteins were found in all lots.

The virus suspension was first concentrated (from 10:1 to a maximum of 100:1) by forced tangential flow through PSVP membranes. The retentate was then washed with 0.1 M phosphate buffer pH 7.3, until constant PN was obtained.

A typical analytical follow-up of the complete cycle is shown in the figure. The whole cycle was carried out in 9 h. Analysis of the retentate obtained after washing showed 96% elimination of PN and a purification factor of 23–40, expressed as the ratio between the final and starting HA/mg PN value. IEP and PAGE control for hens' egg proteins were negative in all lots prepared by this method. The recovery of influenza viruses calculated as HA activity was 100%. In order to find out to what extent it is possible to eliminate residual impurities from membrane-purified vaccine, the retentates were either chromatographed through a Sepharose 2B column (Pharmacia AB Uppsala, Sweden) or ultracentrifuged with a zonal centrifuge (Beckman, type L2-65B equipped with a rotor type CF-32 Ti). The results are shown in the table. With this simple, 1-step concentration and purification method, more than 95% of PN were eliminated, as shown in the 3 lots presented in the table.

It has also been shown that further processing of MF membrane-purified retentate, either with zonal ultracentrifuge or by Sepharose chromatography, results in reduction of PN to less than 1%, in cases where extremely high purity is required. In comparison with other methods used for influenza vaccine concentration and purification, MF membranes with an exclusion limit of 1×10^6 daltons have the advantages of higher yield, more rapid working cycles, and extremely simple management of molecular filtration.

A newly designed decantation for purified synaptic vesicle

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Summary. A new decanting device of fine stainless steel needles has been developed, which can remove a layer from a gradient with little contamination from the adjacent upper or lower layers. This new apparatus can be used for removal of a very narrow band from a density gradient and has been successfully used in the fractionation of synaptic vesicles from brain homogenates.

There are many instruments available for marking the density gradients used in ultra-centrifugal fractionation[1,2]. However, little attention has been paid to decantation and to the problems of contamination between adjacent layers which are encountered with the routine method[3–5]. A new decanting device of fine stainless steel needles is shown in figure 1.

Methods and results. Fine needles of outer diameter 0.4 mm ⌀ were used in the device as shown in figure 1. We have called the device a 'multi-fine needle' decanter. 25 stainless steel needles were gently inserted into the solution in the centrifugation tube (diameter 14 mm) and a layer of the solution was pushed out by applied pressure. The outflow rate could be controlled by altering the pressure by using an O-ring. The contamination made by turbulence around each of the needle tips was reduced to a minimum compared to the conventional methods shown in figure 2. Quantities as little as 0.5 ml could be removed from the tube without contamination. For a layer

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