to a change in metabolic rates after rabies inoculation in these animals. The relatively larger experiments with mice, and the greater number of these experiments suggest a delaying effect to time of onset (due to PTU in the diet) and a consequent higher survival rate.

Summary. Studies were made to determine if raising or lowering the metabolic rate of experimental animals would increase or decrease the incubation period of rabies virus.

The metabolic rate of mice, rats, hamsters, and guinea pigs was increased from 17 to 84% with iodinated casein with no significant change in the incubation period or mortality rate of rabies when compared to control animals. The host metabolic rate was decreased by thyroidectomy, hypophysectomy, or propylthiouracil in the diet. In most cases the incubation period of the treated groups did not differ significantly from the rabies controls. However, in thyroidectomized rats with the metabolic rate decreased 13% there was a significant delay in the onset of rabies symptoms ($p = .10$). In mice fed PTU the mean metabolic rate ranged from a 2.2% increase to a 4% decrease. An analysis of 216 mice suggests a delaying effect in the onset of rabies symptoms and a higher survival rate for mice with PTU in the diet than controls with a normal diet. It is suggested that the decreased metabolic rate of a host could delay virus propagation and give more time for active and passive treatment and possibly increase the chances of survival for an animal exposed to rabies virus.

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Yellow Fever Vaccine I. Development of a Vaccine Seed Free from Contaminating Avian Leukosis Viruses (32885)

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Chicken flocks are known to harbor avian leukosis viruses (ALV) which are transmitted from mother hen to egg. Special conditions of isolation and rearing are necessary to produce ALV-free eggs. Embryos of ALV-infected eggs and live virus vaccines prepared from these embryos are also contaminated. Rigorous control is exercised in the production of vaccines of chicken embryo origin (e.g., measles) and additional careful testing of the final vaccine insures a product which is ALV-free.

Yellow fever (YF) vaccine currently available in the United States is known to be contaminated with ALV. This vaccine is produced on a "seed lot system" (1), i.e., primary seed virus is used to prepare lots of secondary seed which, in turn, is utilized for the preparation of vaccine lots. The primary seed (lot AB237) which is ALV-contaminated was produced in an era (middle 1940's) when little was known of ALV contamination of eggs. In order to make ALV-free YF vaccine, it would be necessary to develop an ALV-free primary seed and to produce all subsequent secondary seeds and vaccines in ALV-free eggs.

Our yellow fever project was initiated with the following objectives: (i) to develop an ALV-free primary seed by ridding the 17D virus vaccine seed of its ALV contaminants, (ii) to determine the safety and immunogenicity of this ALV-free YF virus strain in monkeys, and (iii) to determine its immunogenicity in man. This report deals with the successful accomplishment of the first objective.

Materials and Methods. Viruses. Lot 145-3 (passage 229) of 17D YF virus, a vaccine prepared at Rockefeller Foundation in 1943 and known to be free from human hepatitis virus (2), was used as inoculum for lot 145-3A1,[1] which was employed in our filtration experiments. Although prepared in ALV-free eggs, lot 145-3A1 (passage 230) contained ALV because the inoculum was contaminated. The passage history of 17D YF virus has been described (2–5).

Other YF virus preparations used in the filtration experiments were from the collection maintained at the Arbovirus Unit of this Laboratory.

The RPL 12-L31 strain of RIF (resistance-inducing factor) virus (6) was originally obtained from Dr. Benjamin Burmester, Regional Poultry Laboratory, East Lansing, Michigan. Our studies were performed using a reagent pool prepared in the Avian Leukosis Unit of this Laboratory.

Animals. Suckling (1–3 day old) and weanling (16–20 gm) white mice (General Purpose strain) obtained from the Rodent and Rabbit Production Section, Laboratory Aids Branch, NIH was used for assaying YF virus. Mice were inoculated intracerebrally with 0.03 ml and observed daily for 21 days; LD_{50} was calculated.

The RIF-free embryonated eggs used in these studies were produced by the NIH RIF-free flock of white leghorn chickens.

Tissue culture. The MA-104 embryonic rhesus monkey kidney cell line (Microbiological Associates, Inc., Bethesda, Maryland) was also used for assaying YF virus.[2] Two-oz prescription bottle cultures were inoculated with 0.2 ml and after adsorption for 1–2 hours at 37°C, 5 ml of an agar overlay medium (7) was added. Plaques were counted on days 5 and 6 postinoculation. Chick embryo fibroblasts used to assay ALV by the RIF test procedure were prepared from ALV-free eggs.

ALV assay. The ALV was assayed by RIF, COFAL[3] and fluorescent antibody (FAB) tests according to standardized procedures[4] used in our laboratory.

Filtration. Virus preparations were filtered sequentially through GS (220 mμ), VC (100 mμ) and VM (50 mμ) Millipore membrane filters. A prefilter was always used in conjunction with the GS membrane.

Calculations. All titers were determined by the Kärber method (9) and expressed as Dex[5] values (10).

Results. Filtration of yellow fever and RIF viruses. Three different YF virus preparations were filtered through Millipore membranes (Table I): chicken embryo (CE) derived 17D virus vaccine, suckling mouse brain (SMB) passaged 17D virus, and the French neurotropic (FN) strain of SMB origin. Although the titers of the starting material were higher by approximately 2 Dex in the SMB pools than in the CE preparation, the effects of filtration were similar. Consistently, there was a slight increase in titer after passage through the 220-mμ membrane, possibly due to disruption of aggregates. Also, consistently, there was a slight drop in titer after passage through the 100-mμ membrane, and a drop of approximately 2 Dex after passage through the 50-mμ membrane.

These results indicate that YF virus is small and less than 50 mμ.

The results of filtration of RIF virus show that it is definitely larger than YF virus (Table II) being completely retained by the 50-mμ Millipore membrane.

Filtration of yellow fever virus vaccine. Based on the above results an experiment was set up to attempt removal of the contaminating RIF virus from YF vaccine by

[1] Prepared by National Drug Company under contract by the Division of Biologics Standards.

[2] Spector, S. and Tauraso, N. M., in preparation.

[3] COFAL = complement fixation for avian leukosis (8).

[4] Available on request.

[5] Decimal exponent.

TABLE I. Filtration of Yellow Fever Viruses Through Millipore Membrane Filters.

Virus strain	Pore size of membrane (mμ)	Titer in SM[a]	Drop in titer on passage through membrane
17D	(not filtered)	4.7[e]	
(CE origin)[b]	220	4.8	(+0.1)[f]
	100	4.5	0.3
	50	2.3	2.2
17D	(not filtered)	6.3	
(SMB origin)[c]	220	6.8	(+0.5)
	100	6.7	0.1
	50	4.9	1.8
FN	(not filtered)	6.7	
(SMB origin)[d]	220	7.0	(+0.3)
	100	6.8	0.2
	50	4.4	2.4

[a] SM = suckling mice.

[b] A preparation of 17D Yellow Fever Vaccine, National Drug Co., lot 6374.

[c] A strain of 17D virus originating from a vaccine preparation and subsequently passaged in suckling mice by intracerebral inoculation; suckling mouse brains (SMB) were harvested as mice sickened and prepared as 20% suspensions in phosphate-buffered saline with 0.5% bovine plasma albumin.

[d] The French neurotropic strain of yellow fever virus maintained in our laboratory by serial passages in suckling mice.

[e] Dex value (10) per .03 ml of inoculum.

[f] Positive values indicate an increase in titer.

differential Millipore filtration.

Yellow fever virus, lot 145-3A1, was filtered sequentially through 220-mμ, 100-mμ, and 50-mμ pore size Millipore membranes and the respective filtrates were inoculated into RIF-free eggs. After incubation at 37°C for 72 hours, the living embryos of each group (lots 145-3A1-220, 145-3A1-100 and 145-3A1-50, respectively) were harvested and pools prepared according to the method for vaccine production (11, 12). Table III is a summary of the results of our studies on the state of YF and RIF viruses in these pools. The results indicate that the 50-mμ membrane allowed YF virus to pass through but completely retained RIF virus, thus achieving separation of the two viruses by physical means. Table III also shows the greater sensitivity of the suckling mouse for assaying YF virus, regularly observed for this and other arboviruses.

Subsequently, lot 145-3A1-50 (passage 231) was used to prepare the RIF-free primary seed, lot 17D-51[6] (passage 232); the latter, in turn, was used to prepare a RIF-free secondary seed, lot 6676 (passage 233). A comparison of the RIF-contaminated and RIF-free 17D yellow fever primary and secondary virus seeds shows their titers to be comparable (Table IV).

Discussion. The attenuated 17D strain of YF virus was developed when little was known of ALV infection of eggs. The ALV infection is widespread in conventional chicken flocks, resulting in contamination of eggs and egg derived vaccines. Yellow fever vaccine has been shown to be contaminated with RIF virus (13). It would not be sufficient to produce all new YF vaccine lots in ALV-free eggs because RIF virus in the YF primary vaccine seed would replicate, resulting in RIF-contaminated secondary seeds and final vaccine. What was needed was to produce an ALV-free primary seed and to prepare all subsequent secondary seeds and vaccines in ALV-free eggs.

Several methods were considered for separating viruses in a mixture. The *terminal-*

TABLE II. Filtration of RIF (RPL 12-L31) Virus Through Millipore Membrane Filters.

RIF test no.	Pore size of membrane (mμ)	Titer per ml	Drop in titer on passage through membrane
1	(not filtered)	6.5[a]	
2	220	5.5	1.0[a]
3	100	1.5	4.0
4	50	0	
5	(neg RIF control)	0 [b]	
6	(pos RIF control)	+[b]	

[a] Dex value (10).

[b] RIF test controls are designated as being positive or negative.

[6] A lyophilized portion of this lot is designated 17D-51A; a wet-frozen portion is designated 17D-51-BC.

TABLE III. Yellow Fever and RIF Viruses in Pooled Suspensions of Chick Embryos Inoculated with Millipore-filtered Virus.

Pool designation	Size of filter inoculum for pool passed through (mμ)	YF virus titer		RIF virus tests		
		WM[a]	SM	RIF	FAB	COFAL
145-3A1	None	4.7[e]	6.0	+	+	+
145-3A1-220[b]	220	6.6	7.2	+	+	+
145-3A1-100[c]	100	5.6	6.6	+	+	+
145-3A1-50[d]	50	5.3	6.0	0	0	0

[a] WM = 16–20 gm weanling mice inoculated intracerebrally with 0.03 ml of appropriate dilutions; SM = suckling mice inoculated similarly.

[b] Pool of chick embryos inoculated with 145-3A which passed through 220-mμ Millipore filter.

[c] Same as [b] except that the inoculum passed through 100-mμ Millipore filter.

[d] Same as [b] and [c] except that the inoculum passed through 50-mμ Millipore filter.

[e] Dex value (10) per 1.0 ml of inoculum.

TABLE IV. Comparison of RIF-contaminated and RIF-free 17D Yellow Fever Vaccine Pools.

Type of pool	Lot no.	Passage no.	RIF status	Titer		
				LD_{50}		pfu[a]
				WM[b]	SM[b]	
Primary seed	AB237	232	contaminated	6.6[c]	7.2	6.4
	(17D51-A)	232	free	6.3	6.8	5.8
Secondary seed	5731	233	contaminated	5.5	5.9	4.9
	(6676)	233	free	6.4	6.3	5.7

[a] pfu = plaque forming units.

[b] WM = weanling mouse; SM = suckling (1–3 day) mouse.

[c] Dex value (10) per 1.0 ml.

dilution method is unsuitable because ALV grows to a titer 1 to 2 Dex higher than YF;[7] this method could eventually lead to preparing ALV free from YF virus. *Plaque purification* is also unsuitable because the only cell culture system which can be used is primary chick embryo. The ALV is present at a higher titer than YF virus[7] in the vaccine preparations and many plaques would have to be selected before achieving separation by this method. Besides, little is known about the homogeneity of the 17D strain virus population and the selection of a single plaque might risk selecting a less attenuated strain or one that is too attenuated. *Antibody neutralization* of one virus in a mixture can result in growth of the other virus in the mixture. Other methods, such as *differential ether and heat sensitivity, selective analogue antagonism, and inorganic cation selective suppression,* were also considered unsuitable.

The experiments described in this paper show yet another way of separating 2 viruses in a mixture. Differential filtration through membrane filters of different pore sizes can achieve purification of the smallest of 2 or more viruses in a mixture (14). Fortunately, YF virus is considerably smaller than ALV; this allows purification by physical means which offers definite advantages. Firstly, the purified virus in the filtrate should be more representative of the original virus population than if terminal dilution or plaque purification were employed. Secondly, avoiding the use of other reagents of dubious purity, such as ALV antibody of avian origin, eliminates the risk of contaminating the virus seed with still

[7] Unpublished results.

other agents. Thirdly, the separation is achieved at a single passage level. This eliminates the need for additional passages which could result in further changes in the virus. Our ALV-free primary and secondary vaccine seeds are at the same passage levels as the current ALV-contaminated primary and secondary vaccine seeds.[8]

Comparative studies of the ALV-contaminated and ALV-free vaccine seeds with respect to neurovirulence tests in monkeys and immunogenicity in monkeys and man are underway.

Summary. An avian leukosis virus (ALV) free primary yellow fever vaccine seed has been developed by ridding the 17D virus vaccine seed of its ALV contaminant. By differential filtration through Millipore membrane filters of different pore sizes, yellow fever virus was separated from its contaminant by physical means. The ALV could not be detected in the new primary and secondary vaccine seeds by RIF, COFAL, and FAB tests.

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[8] An updated passage history of the YF vaccine virus will be published elsewhere. In brief, the passage history of the ALV-contaminated primary seed is: lot 145-3 (passage 229) → lot YF-1 (P230) → lot YF 10 (P231) → lot AB237 (232); for the ALV-free primary seed the history is: lot 145-3 (P229) → lot 145-3A1 (P230) → lot 145-3A1-50 (P231) → lot 17D-51 (P232).

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A Note on the Intestinal Absorption of Cholesteryl Ethers in the Rat* (32886)

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It has been reported that cholesteryl-2,2-methyl ethyl caproate, the ester bond of which is not split by cholesterol esterase *in vitro* is not absorbed by the intestinal tract of the rat (1). This evidence was based on the absence of this ester in the thoracic duct lymph when fed to rats. The above finding was interpreted by Vahouny and Treadwell (1) to show what they call an "absolute requirement for free sterol for absorption by rat intestinal mucosa."

Napier (2) found "no significant" absorption of cholesterol esters of 2-methyl laurate or myristate in balance studies in the rat. Significant amounts of the cholesterol moiety of these esters (maximally 3.6%) were,

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