those exhibited by rats receiving both orotic acid and choline. No alternate explanation of the effect of orotic acid on the neutral fat content of the rat liver is apparent at this time.

Summary. 1. Inclusion of orotic acid in the diet of young rats results in fatty liver formation. 2. Uracil and thymine, known to be synthesized from orotic acid, are without effect on liver fat content. 3. This effect of orotic acid is not counteracted by lipotropic factors such as folic acid, cobalamine, methionine or choline.

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Received September 16, 1955. P.S.E.B.M., 1955, v90.

Production of Type Specific Antisera to Poliomyelitis Viruses in Rabbits.* (22006)

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The objective of this study was the production for experimental purposes of anti-poliomyelitis sera of high titer, with a high degree of type-specificity. The report of Hirst and Gotlieb(1) of the production of highly specific antisera to influenza viruses by intravenous injection of rabbits suggested that this approach might well serve our purpose. Levinson *et al.*(2) have reported the production of antisera capable of neutralizing poliomyelitis viruses, by intravenous or intramuscular injection of rabbits. They refer to earlier experience of other investigators in producing such neutralizing antibody in the serum of non-susceptible animals.†

The results reported here consist of an extension of the findings of Levinson's group, by the use of smaller doses of virus as antigen given by the intravenous route. In addition, a direct effect of the antisera alone on kidney cell culture has been observed. The main objective was achieved by repeated intravenous injections of rabbits with small doses of each of two types of poliomyelitis viruses grown in tissue culture. The titer of antibody to the same type of virus as that used for antigen (homotypic) as well as to the other type of virus (heterotypic) have been determined by neutralization tests in tissue culture.

Materials and methods. Type 1-Brunhilde and Type 2-YSK poliomyelitis viruses (obtained from Dr. Joseph L. Melnick, Yale University) which had been grown in either monkey testicular or human uterine tissue culture were used as antigens to start immunization of rabbits and for a booster dose at 5 months. The 50% endpoints (negative logarithm) of the cytopathogenic action of the viruses in HeLa culture were approximately 5 and 4 respectively. For booster doses, at 6 months or more from the beginning of immunization, Type 1-Mahoney and Type 2-MEF1 viruses grown in monkey kidney culture (obtained from Connaught Laboratories, Toronto, Canada) were used. The 50% endpoints of viral action in monkey kidney culture were 6.4 and 5.3 respectively. Supernate from a lightly centrifuged culture served as

* This work was aided by a grant from the National Foundation for Infantile Paralysis.

† By personal communication, Dr. Hubert Malherbe in the laboratory of Dr. John F. Enders has also reported the production of neutralizing antisera to poliomyelitis viruses, by intramuscular injection of rabbits with virus incorporated in oily adjuvants.

TABLE I. Development of Type 1 Neutralizing Antibody in Serum of Rabbits after Repeated Intravenous Injections of Type 1 Poliomyelitis Virus.

Interval since beginning immun. (mo):	1	5¾	6¾	8¼	14⅔	15
" " last booster (wk):	—	3	2	1	—	1½
Total No. of doses:	6	7	8	9	9	10
	Reciprocal of dilution of serum at 50% endpoint of viral effect					
Rabbit No. 10	20	30	400	1800	Dead	
11	20	5	110	600	14	180
12	20	Dead				
14	30	330	380	1600	140	680
Log_{10} ED_{50} Mahoney virus in test:	2.4	2.3	1.9	2.1	2.0	2.0

antigen for injection. In neutralization tests, the Mahoney and MEF1 viruses were used. Equal parts of virus and serum dilution to be tested were mixed and incubated for one hour at 35°C. 0.1 ml of the mixture was added to 0.5 ml nutritive medium in a tube containing one-week-old monkey kidney cells cultured directly on glass(3). (Screw-capped pyrex glass tubes which had been inoculated with approximately 50000 monkey kidney cells were received from Microbiological Associates, Bethesda, Md.) Occasionally, confirmatory tests were made in HeLa cell culture(5), usually 4 days after inoculation of pyrex glass test tubes with approximately 30000 cells. Final observation of viral action was made at 5 or 7 days, depending on the condition of the control cultures, without added virus. The amount of virus introduced in all but one neutralization test was within the range of 50 to 250 ED_{50} (effective doses); *i.e.*, $10^{1.7}$ to $10^{2.4}$ times the amount of virus at the 50% endpoint of cytopathogenic action of virus in simultaneous titration in normal rabbit serum. Dilutions of virus in titration and of antiserum for determining neutralization endpoints were made at 0.5 log increments. Titrations were done in 3 to 6 replicate tubes. 50% endpoints have been calculated according to Reed and Muench(4). Serum endpoints are reported as the dilution of antiserum that was mixed with an equal volume of virus. Some values given in the tables represent the geometric mean of endpoints found in 2 or 3 tests done on different days.

Immunization schedule. New Zealand white rabbits weighing 2-3 kg were injected intravenously. Two groups of 5 rabbits each were given 6 doses of 2 ml tissue culture virus of Type 1 or Type 2 as 3 doses a week for 2 weeks. At 5 months from the beginning of immunization, a booster dose of 1 ml was given. At approximately 6, 7 and 8 months, 2 ml each, and at 15 months, 3 ml booster doses of virus grown in monkey kidney culture were given. The rabbits were bled by cardiac puncture before, and 1 month after the beginning of immunization. They were bled also from 1 to 3 weeks after each booster dose. No anaphylactic reactions were observed. The rabbits continued to gain weight during the course of immunization. Deaths which occurred were not related to time of injection.

Results of homotypic neutralization tests. In each neutralization test, a "normal" serum control was included. This consisted of several pools of serum from the same rabbits taken before immunization, mixed with the same amount of virus as in the test. When the normal serum pool was tested "undiluted" there was no evidence of neutralization. A more sensitive test for the presence of any neutralizing capacity of pre-immunization serum consisted of titration of virus mixed with undiluted serum, either compared simultaneously with virus titrated in Earle's solution or with the expected titer based on 21 titrations with Mahoney, and on 22 titrations with MEF1 virus. With Mahoney virus, 5 separate titrations in monkey kidney culture and with MEF1 virus, 4 titrations in monkey kidney (and 1 in HeLa culture) yielded the expected, or observed, equal titer. Thus there was no evidence of any neutralizing effect of the serum of these rabbits before immunization.

TABLE II. Development of Type 2 Neutralizing Antibody in Serum of Rabbits after Repeated Intravenous Injections of Type 2 Poliomyelitis Virus.

Interval since beginning immun. (mo):	—	1	5½	6½	8	14⅔	15
" " last booster (wk):	—	—	3	2	1	—	1½
Total No. of doses (2 ml each):	0	6	7	8	9	9	10
	Reciprocal of dilution of serum at 50% endpoint of viral effect						
Rabbit No. 2		30	280	1700	5600	Dead	
15	0	100	300	5000	2500	400	560
16	0	10	350	2800	2000	560	1000
17		30	Dead				
18		100	"				
Log_{10} ED_{50} MEF1 virus in test:	1.8	1.9	2.0	1.9	1.8	1.8	1.8

Table I shows the neutralizing capacity of anti-Type 1 serum vs. homotypic Mahoney virus. Antibody was demonstrable in all 4 rabbits at 1 month, the level rising with 3 booster doses up to 8¼ months after the beginning of immunization when endpoints of serum of the 3 surviving rabbits ranged from 600 to 1800. During a subsequent rest period of 6 months, endpoints fell although antibody was still demonstrable. A subsequent booster dose in the 2 remaining rabbits raised the endpoints to moderately high levels, although not quite up to the maxima observed at 8 months.

Table II gives the endpoints of neutralization of anti-Type 2 sera vs. homotypic MEF1 virus. All 5 rabbits showed an antibody response of from 10 to 100 by the end of the first month. With 2 or 3 subsequent booster doses, levels rose to give endpoints at 8 months of from 2000 to 5600. After a rest period of nearly 7 months, antibody fell to moderate levels in the 2 surviving rabbits and rose somewhat again after one more antigenic stimulus.

Results of heterotypic neutralization tests. Table III gives the results of cross neutralization tests. The sera of 3 rabbits before immunization with Type 2 poliomyelitis virus showed no effect on Type 1 virus. By 6½ months after beginning of immunization, after 2 booster doses, sera of 2 of 3 rabbits showed endpoints of 10 each, the third, 0. An additional booster by 8 months did not increase this cross reaction. (The homotypic endpoint ranged from 2000 to 5600 in these three samples of serum.) None of 3 anti-Type 1 antisera (see Table III) showed heterotypic antibody by nearly 6 months, after 6 doses of virus plus a booster dose. After a second and third booster dose, at approximately 7 and 8 months, 1 (or possibly 2) of 3 sera showed a minimal amount of cross neutralization. The homotypic endpoints of these sera at 8¼ months ranged from 600 to 1800. Later samples of sera were not tested vs. heterotypic virus because of a complicating reaction which will be described in the next section.

Immuno-cytopathogenic effect of antiserum. Since the initial series of injections as well as the first booster dose in the rabbits consisted of virus grown in human uterus or in monkey testis, the possibility of a reaction of the antisera directly on the monkey kidney cells was not as great as if the virus had been grown in the same type of cells as that used in the neutralization test. Indeed, the antisera being studied for antiviral activity showed no direct effect on monkey kidney cells until the 7 and 8 months' samples, after the rabbits had received their first exposure to virus grown in monkey kidney culture as 1 and 2 additional booster doses. The serum of one (#10) of the 6 rabbits remaining showed cytopathogenic effect at a 1/6 final dilution of serum; *i.e.*, 0.1 ml undiluted serum added to 0.5 ml culture fluid. After the third booster dose of monkey kidney grown virus at 15 months, sera of all 4 rabbits surviving produced an untoward effect on monkey kidney cells in culture. The reaction consisted of either lysis of agglutination of the cells, resulting in the extreme in their complete destruction or their falling off the wall of the test tube. The reaction is comparable to that observed when HeLa cells were exposed to antiserum prepared in rabbits by intravenous injection of HeLa cell constituents(6). The

TABLE III. Degree of Cross Type Neutralization of Anti-Poliomyelitis Sera Produced in Rabbits.

		Anti-Type 2 serum vs. Type 1 (Mahoney) virus				Anti-Type 1 serum vs. Type 2 (MEF1) virus			
Interval since beginning immun. (mo):		—	5½	6½	8		5¾	6¾	8¼
" " last booster (wk):		—	3	2	1		3	2	1
Total No. of doses:		0	7	8	9		7	8	9
	Rabbit No.					Rabbit No.			
	2	0	0	0	0	10	0	10	3
	15	0	0	10	2	11	0	2	0
	16	0	10	10	10	14	0	0	0
Log_{10} ED_{50} virus:		2.1	1.7	2.2	2.4		1.7	1.9	2.8

addition of neutral red at a final dilution of 1/100,000, observed to be compatible with healthy cells, helped to visualize the reaction. One antiserum (rabbit #14, 15 months' sample) was tested repeatedly, yielding an average 50% endpoint of 30 of anti-kidney cell reaction. The endpoint of homotypic virus neutralization by this serum was 680.

The possibility of a cytopathogenic action on cells in culture not due to virus must be taken into account when carrying out neutralization tests, especially with high concentrations of antiserum such as used in heterotypic tests. Unless the antiserum alone is shown to have no effect on the cells in culture, this anti-cellular reaction could be mistaken for lack of cross neutralization.

Summary. A simple and inexpensive method for producing antisera with a high degree of neutralizing capacity for poliomyelitis viruses has been described. By repeated intravenous injection of rabbits with small doses of poliomyelitis viruses grown in tissue culture, antisera with a high degree of homotypic with little or no heterotypic neutralizing capacity have been produced regularly. Such antisera develop also to a lesser degree the capacity to lyse or agglutinate the cells in culture, in the absence of virus. When testing for heterotypic neutralization, this reaction may mask cross neutralization.

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