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9. After the present study was completed we discovered independent confirmation of our results in studies by J. Cosnier [*Compt. Rend. Soc. Biol.* **157**, 1611 (1963)] and J. Cosnier and C. Couturier [*ibid.* **160**, 789 (1966)] who used exposures of newborn for 2 hours a day to induce maternal behavior in intact and ovariectomized nonpregnant females. The latencies that they observed appear to be similar to the ones found by our method.

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Altered Response to Pneumococcal Polysaccharide in Offspring of Immunologically Paralyzed Mice

Abstract. *The dose of pneumococcal polysaccharide type III required to induce immunologic paralysis in newborn offspring of immunologically paralyzed mice was one-tenth of the corresponding paralyzing dose for newborn offspring of normal mice. Similarly, immunization of the offspring of the paralyzed mice was accomplished with one-tenth the dose of polysaccharide necessary to immunize normal newborn mice. The altered susceptibility of newborn mice from paralyzed mothers to the induction of both paralysis and immunity was predicted from theories of antibody formation which postulate that the induction of tolerance or immunity is controlled by the concentration of natural antibodies specific for the antigen used.*

The theory of antibody formation proposed by Eisen and Karush (*1*) postulated, as did that by Jerne (*2*), that preformed natural antibody functions as the immunologic recognition system. According to Eisen and Karush (*1*) bimolecular antigen-antibody complexes (AgAb) constitute an effective stimulus for antibody formation and result in immunity. Trimolecular complexes (Ag_2Ab) would not stimulate the antibody-producing cells and would result in immunologic paralysis. The latter complexes are assumed to be degraded and eliminated from the organism. The induction of paralysis or immunity in the animal would depend upon the ratio of the concentration of antigen to that of the corresponding natural antibody present. The paralyzed animal would not have in circulation natural antibody specific for the antigen, since this antibody would have been removed after forming a complex with excess antigen.

Support for this view was provided by the work, of Segre and co-workers (*3*), on the enhancement by specific antibody and by normal γ-globulin of the antibody response in pigs deprived of colostrum. Dawe *et al.* (*4*) presented evidence that the enhancement of immunologic reactivity in rabbits injected with Freund's adjuvant was due, in part, to an increase in concentration of natural antibody. Hemphill *et al.* (*5*) succeeded in terminating immunologic tolerance to human serum albumin in mice by exposing their spleen cells to antigen-antibody complexes, but not to antigen alone. We now present evidence that the susceptibility of newborn mice to the induction of immunologic paralysis and immunity with pneumococcal polysaccharide is controlled by the concentration of natural antibodies specific for the polysaccharide.

Newborn mice acquire maternal γ-globulin both by transplacental transfer and by intestinal absorption from the colostrum during the first days of life (*6*). Since natural antibodies are presumably γ-globulins, the concentration of natural antibodies in the circulation of newborn mice should be the sum of the individual's own antibodies and those passively acquired from the mother. Thus, if a newborn mouse were the offspring of an immunologically tolerant mother it would have in circulation only its own natural antibody with specificity corresponding to the tolerated antigen. The concentration of natural antibody against the tolerated antigen should be lower in the offspring of a tolerant mother than in the offspring of a normal mother.

Adult female white mice (NIH) were immunologically paralyzed by intraperitoneal injection of 500 μg of pneumococcal polysaccharide type III dissolved in sterile saline (*7*). Preliminary experiments indicated that this dose of polysaccharide resulted in immunologic paralysis, as reported also by Felton (*8*). Both normal untreated and treated adult female mice were bred. Within 24 hours after birth, newborn from both paralyzed and normal mothers were injected intraperitoneally with varying quantities of the polysaccharide. The mice were challenged 7 days later with 100 minimal lethal doses (MLD) of the infectious tissue suspension (*7*) to determine the immunizing and paralyzing doses for each group (Table 1).

It was found that offspring of normal mothers were immunized with 0.05 μg and paralyzed with 0.5 μg of the polysaccharide. In contrast, the newborn from mothers paralyzed with the polysaccharide were paralyzed with 0.05 μg of the polysaccharide (a dose which immunized the offspring of normal mothers) and were immunized with 0.005 μg. The differences in the responses of the two groups of mice are consistent with the assumed differences in the quantities of natural antibody available to act as part of the immunologic recognition system. The reduction in the quantity of antigen necessary to induce paralysis or immunity appears to reflect a reduction in the quantity of natural antibody.

The possibility of transplacental transfer of the polysaccharide was also investigated. Pneumococcal polysaccharide present in the mother could pass through the placenta and influence the responsiveness of the newborn. If this were the case, the results for newborn from paralyzed mothers (Table 1) could be explained by the fact that enough of the polysaccharide was transferred to the fetus to reduce markedly the dose of antigen necessary to induce paralysis. It was, therefore, desirable to determine whether transplacental transfer of the polysaccharide took place.

Table 1. Titration of paralyzing and immunizing doses of pneumococcal polysaccharide in adult and newborn mice.

Mice (No.)	Antigen (μg)	Survivors	
		No.	%
	Adults		
6	500	0	0
6	100	0	0
6	10	0	0
6	1*	1	16
6	0.5†	6	100
6	.05	1	16
6	.0	0	0
	Newborn from normal mothers		
6	5.0	0	0
6	0.5*	1	16
6	.05†	6	100
6	.005	0	0
6	.0	0	0
	Newborn from mothers paralyzed with pneumococcal polysaccharide		
37	0.05*	2	5.5
36	.005†	35	97
11	.0005	1	9

* Minimal paralyzing dose. † Minimal immunizing dose.

Paralyzed pregnant mice were killed 19 days after they were bred. The embryos were removed aseptically and homogenized in sterile saline. Doses of homogenate ranging from 0.15 to 3.15 embryo equivalents (the number of embryos used to obtain a given quantity of homogenate) were injected intraperitoneally into adult mice. Newborn mice received doses of homogenate ranging from 0.1 to 0.63 embryo equivalent. The mice were then challenged with 100 MLD of infectious tissue suspension and 1 week after injection of the homogenate all succumbed (Table 2). Since the mice did not survive challenge, the quantity of polysaccharide present in the embryonic homogenates may have been either too little to immunize, or large enough to induce immunologic paralysis. From the dose of polysaccharide known to immunize newborn mice (0.05 μg) it can be calculated that, if each embryo had contained as much as 0.08 μg of polysaccharide, the newborn mice that received 0.63 embryo equivalent (Table 2) should have become immune. Similarly, from the dose of polysaccharide required to paralyze adult mice (5 μg) it can be calculated that, if each embryo had contained as much as or more than 33.3 μg of polysaccharide, the adult mice which received 0.15 embryo equivalent (Table 2) could have been immunologically paralyzed. However, this quantity of polysaccharide should have been detectable by the phenol–sulfuric acid method (*9*) which had a threshold sensitivity equal to 20 μg of polysaccharide. In fact, no polysaccharide was found by the phenol–sulfuric acid method in the embryonic homogenate or in an extract prepared from it by Heidelberger's method (*7*). It can, therefore, be concluded that, if transplacental transfer of the polysaccharide occurred at all, it could only account for less than 0.08 μg of polysaccharide per embryo. It appears unlikely that this quantity of polysaccharide was sufficient to induce paralysis of the embryos *in utero* and thus account for the low dose of antigen required to induce paralysis in the newborn offspring of paralyzed mice. Furthermore, even if this interpretation were accepted, it would be difficult to explain on the same basis the increased susceptibility of the offspring of paralyzed mice to the induction of immunity, unless one assumed that the transferred polysaccharide induced simultaneously sensitization of some, and paralysis of some other, cells of the embryos. The dose of polysaccharide administered at birth would then determine whether paralysis or immunity ensued. The simultaneous occurrence of paralysis and immunity was claimed by Mitchison (*10*) in connection with experiments in which a double threshold of immunologic paralysis to bovine serum albumin in mice was reported. Siskind and Howard (*11*), however, did not find a double threshold of paralysis in mice tolerant to pneumococcal polysaccharide. The results reported here are not readily explained by assumption of transplacental transfer of polysaccharide in quantities too low to be detected. If enough antigen were transferred transplacentally to paralyze the mice *in utero*, the administration of an additional 0.005 μg of antigen at birth should not result in immunity.

Table 2. Failure of homogenates of embryos from immunologically paralyzed mice to immunize adult and newborn mice against challenge with pneumococcus.

Status of mice	Embryo equivalents*	No. survivors / No. in group	Survivors (%)
	Test		
Adult	0.15	0/6	0
Adult	1.0	0/10	0
Adult	2.0	0/5	0
Adult	3.15	0/10	0
Newborn	0.1	0/24	0
Newborn	0.2	0/10	0
Newborn	0.63	0/10	0
	Control		
Adult	0.5 μg†	12/12	100
Newborn	0.05 μg†	17/17	100

* The number of embryos used to prepare the quantity of homogenate injected. † Control mice received the indicated quantity of pneumococcal polysaccharide.

Increased susceptibility of the offspring of tolerant animals to the induction of tolerance has been reported. Hort (*12*) demonstrated that the offspring of Peking ducks made tolerant by neonatal injection of muskovy duck spleen cells reacted to a lesser degree to skin grafts from muskovy ducks than did offspring of normal ducks. In addition, the experimental ducks developed lower hemagglutination titers against muskovy duck erythrocytes than did the control ducks. Argyris (*13*) showed that the offspring of C_3H mice made tolerant to CBA skin grafts were more susceptible to the tolerance-inducing stimulus of CBA spleen cells than were offspring of normal C_3H mice. Although these results may be explained by the failure of tolerant dams to transfer antibodies specific for the tolerated antigens to their offspring, they can also be interpreted on the assumption that the offspring were exposed to the tolerated antigens *in ovo* or *in utero*, thus becoming partially tolerant before hatching or birth. In contrast, our experiments are open only to the former interpretation, and are consistent with models of antibody formation which locate the immunologic recognition system at the level of circulating natural antibodies (*1, 2*). Interpretation of our results on the basis of the clonal selection theory (*14*), which sssumes that cells potentially competent to respond to the tolerated antigen are absent or suppressed in the tolerant animal, does not appear feasible.

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"Galactose Dehydrogenase," "Nothing Dehydrogenase," and Alcohol Dehydrogenase: Interrelation

Cuatrecasas and Segal (*1*, *2*) recently suggested the existence of an interesting new pathway of galactose oxidation in liver tissue from various mammals. They also investigated the electrophoretic mobility of the first step of this pathway, "galactose dehydrogenase," in various species and during development. I have confirmed their experimental results but suggest that the enzyme responsible for the effects observed is really alcohol dehydrogenase (*Enzyme Nomenclature*: Alcohol:NAD oxidoreductase 1.1.1.1.), and the substrate is alcohol, contaminating galactose and other reagents.

I was initially struck by the similarity in the electrophoretic bands, reported by Cuatrecasas and Segal, to "nothing dehydrogenase." Shaw and Koen (*3*) had suggested that "nothing dehydrogenase" activity really represented alcohol dehydrogenase. Using the system described by Cuatrecasas and Segal I have been able to demonstrate that the band that they interpret as galactose dehydrogenase appears with equal intensity if galactose is omitted from the reaction mixture (Fig. 1). I have determined that commercially available hydrolyzed starch (Connaught) contains substantial amounts of alcohol, as measured with crystalline, alcohol-free, yeast or liver alcohol dehydrogenase and NAD (nicotinamide adenine dinucleotide). This alcohol can be removed simply by washing the starch in cold buffer; the activity appears in the washings and disappears after drying at 80°C. Phenazine methosulfate also contains small amounts of alcohol, as measured by the reduction of dichloroindophenol in the presence of crystallized liver alcohol dehydrogenase and NAD. However, only very weak bands or no bands of "nothing dehydrogenase" appear when electrophoresis is carried out on gels prepared from washed starch. These bands are greatly enhanced when alcohol is added to either the starch suspension or the staining mixture in a concentration of 2 m*M*.

Since the incorporation of galactose in the staining mixture failed to result in the appearance of a unique band, and because of my growing realization of the extent of contamination of high-grade reagent chemicals with alcohols, the supernatant fraction of crude homogenates and partially purified enzyme was further studied to investigate the possibility that "galactose dehydrogenase" was really alcohol dehydrogenase.

When galactose, NAD, buffer, and liver-homogenate supernatant are incubated together in the system described (*1*), an increase in optical density at 340 mμ is readily observed. The rate of reduction of NAD in my hands has been very similar to that reported (*1*). However, the following observations lead me to the conclusion that the observed reduction of NAD reflects the action of alcohol dehydrogenase on alcohol rather than the presence of a galactose dehydrogenase:

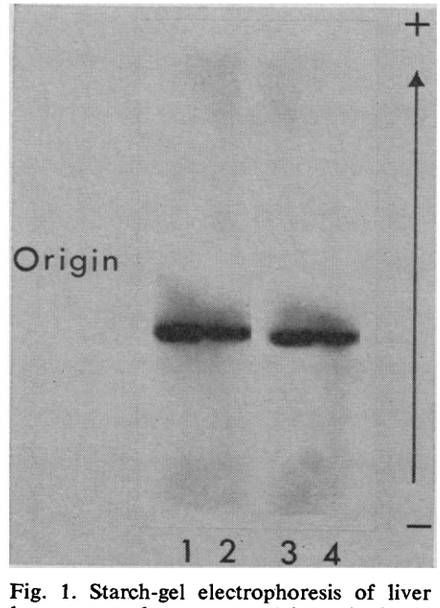

Fig. 1. Starch-gel electrophoresis of liver homogenate from mouse (channels *1* and *3*) and rat (channels *2* and *4*) by use of the system described for staining "galactose dehydrogenase" (*2*). Galactose was omitted from the staining mixture used for channels *3* and *4*.

1) I have found that the most highly purified, commercially available galactose preparations contain ample substrate for commercial, crystalline, horse-liver or yeast-alcohol dehydrogenase. One lot of galactose (essentially glucose-free; Sigma) was found to contain 0.6 mmole of substrate for alcohol dehydrogenase per mole of galactose, and the highest-grade galactose offered by Calbiochem contained 0.8 mmole of alcohol per mole of galactose.

2) The substrate for alcohol dehydrogenase that contaminates galactose is readily removed by drying a 1*M* solution of galactose at 80°C and reconstituting it with distilled water. No "galactose dehydrogenase" activity is found in liver when substrate subjected to this simple drying treatment is used, but there is no destruction of galactose, as measured with the galactose oxidase system (*4*).

3) The alcohol dehydrogenase activity of rat liver is readily partially purified by adsorption of extraneous proteins by treatment with diethylaminoethyl cellulose in 0.005*M* tris-HCl buffer, *p*H 8.8, and taking a 60- to 80-percent ammonium sulfate cut. During this approximately 20-fold purification, alcohol dehydrogenase shows activity with galactose that has not been subjected to drying at 80°C, but no activity with galactose so dried. The ratio of activity with untreated galactose to activity with ethanol remains unchanged throughout purification, and is the same as that found with crystalline horse-liver alcohol dehydrogenase.

4) The rate of reaction of the supernatant from rat liver with galactose is essentially identical with the rate found when the quantity of ethanol, equivalent to the amount of substrate for alcohol dehydrogenase contaminating the galactose, is used.

It seems apparent, therefore, that the "galactose dehydrogenase" activity of mammalian liver is really alcohol dehydrogenase activity acting on alcohols contaminating the reagents used. The highest-quality reagents commercially available to the biochemical laboratory today are contaminated with sufficient quantities of primary alcohols