agreement with those of Stadie and coworkers (1,2). We have been unable to demonstrate either the permanent fixation of bound insulin-I^{131} or a limiting value for bound insulin-I^{131} within the range of solubility of insulin.

It is not the intent of the present report to deny that the biologic effects of insulin are exerted through combination with some cell-fixed enzyme system or substrate compound. It is generally accepted that a combination of some sort is necessary for a chemical reaction between substances to take place. However, the present studies do not support the concept that the binding of insulin-I^{131}, observed *in vitro* with isolated rat diaphragm is necessarily of any physiological significance, or that these observations reflect, in any way, a biologic process which takes place *in vivo*.

If a brief exposure of diaphragm to insulin suffices to augment glycogen accumulation on subsequent incubation with glucose(1), the results of the present study would then suggest that survival time of the altered state induced by insulin must persist appreciably beyond the time of reaction with insulin. An evaluation of this survival time would appear to be of interest.

Summary. Serum albumin, serum γ globulin, crystalline glucagon and crystalline insulin were observed to bind to diaphragm *in vitro* and to be eluted slowly on continuous washing with buffered solutions. A lack of reproducibility of binding of insulin at low concentrations is attributable in part to variable adsorption to glassware. A definite saturation of binding sites on diaphragm was not observed at the highest concentrations employed. Competitive inhibition was observed in the presence of heterologous proteins. Binding capacity was not altered by treatment of diaphragms with formaldehyde. Binding of insulin by isolated rat diaphragm *in vitro* is not demonstrably of biologic significance but is attributable to nonspecific surface adsorption of proteins.

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1. Stadie, Wm. C., Haugaard, N., Marsh, J. B., and Hills, A. G., *Am. J. Med. Sc.,* 1949, v218, 265.

2. Stadie, Wm. C., Haugaard, N., and Vaughan, M., *J. Biol. Chem.,* 1952, v199, 729.

3. Ferrebee, J. W., Johnson, B. B., Mithoefer, J. C., and Gardella, J. W., *Endocrinology,* 1951, v48, 277.

4. Kallee, E., *Z. Naturforsch,* 1952, v76, 661.

5. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., and Newerly, K., *J. Clin. Invest.,* 1956, v35, 170.

6. Pressman, D., and Eisen, H. N., *J. Immunol.,* 1950, v64, 273.

7. Berson, S. A., Yalow, R. S., and Volk, B. W., *J. Lab. and Clin. Med.,* Feb. 1957.

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Susceptibility of Inbred Mice to Group A Streptococcal Infection.* (23076)

WILLIAM H. MURPHY, JR., AND DENNIS W. WATSON
Department of Bacteriology and Immunology, University of Minnesota, Minneapolis.

Group A streptococci isolated directly from patients often are rich in M protein but avirulent to mice unless adapted by rapid successive transfer(1). This study was undertaken to compare susceptibility of mice of diverse genetic constitution to infection by representative group A streptococci. The objective of the investigation was to provide a sensitive experimental host-parasite system that could serve as a more adequate model for study of group A streptococcal infections of man and their late sequelae.

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Materials and methods. Inbred mice used differed significantly in genetic, anatomic, and physiologic constitution(2). BALB, C58,

and a substrain of DBA/2 mice from colonies maintained in our laboratory, and BALB/c, C57 Black (C57-Bl), and AKR mice from Jackson Memorial Laboratory, were 4 to 6 weeks old when used unless specified otherwise. Pen-bred Swiss albino mice, Tumblebrook strain, were included for comparative purposes. *Strains of group A streptococci* grown in Todd-Hewitt broth† at 37°C for 6 hours were diluted decimally in Todd-Hewitt broth maintained at 4°C. Organisms, 0.5 ml, inoculated intraperitoneally into test mice, observed 3 to 4 months, were quantitated by plate counts. The LD_{50} of organisms for mice, calculated by the method of Reed and Muench(4), was defined as number of organisms/0.5 ml of inoculum that killed 50% of test mice in 10 days. Not less than 12 mice were used for each dilution of test organism. Group A streptococci recovered from mice were typed to assure accurate identification.

Results. Susceptibility of inbred mice to group A streptococcal infection. Selection of a strain of *Streptococcus pyogenes,* type 18, to assess susceptibility of mice to infection was arbitrary. Table I reveals that test strains of inbred mice were extraordinarily susceptible to infection; commonly less than 10 organisms caused death. Because of limitations inherent in plate counts and LD_{50} calculations the precise minimal number of organisms required to kill mice was not analyzed. When these survey experiments were extended, it was observed that BALB female mice survived inocula lethal to males in 24 hours. *The possibility that sex or/and age influenced susceptibility of mice to infection* was explored by comparing susceptibility of BALB, Swiss, C58 and DBA 2 mice to infection by *S. pyogenes,* type 18. It is evident from Table II that: a) adult BALB females were significantly more resistant to infection than males, b) both male and female DBA/2 and C58 mice were highly susceptible to infection and c) both age and sex influenced resistance of Swiss mice to infection. *BALB male mice were used to assay pathogenicity of group A streptococci of diverse origin.* A total of 4 strains of *S. pyogenes,* type 18, were included in the test group. The results of this experiment (Table III) were conclusive: the pathogenicity of group A streptococci for inbred mice was not general but limited to a specific strain of a single serotype; organisms freshly isolated from clinical specimens were avirulent. *Host-parasite relationships resulting from infection of inbred mice by S. pyogenes,* type 18. Ideally, animals infected experimentally should provide a model that reflects accurately the host-parasite relationship in infections of man. An important feature of infection of BALB and C58 mice by *Streptococcus pyogenes,* type 18, was the spectrum of diseases produced. For example, male BALB mice, highly susceptible to infection,

TABLE I. Comparative Susceptibility of Inbred Mice to Infection by *Streptococcus pyogenes,* Type 18.

Strain of mice*	LD_{50}
Swiss	$10^{5.14}$
BALB	$<10^{1}$
BALB/c	"
C58	"
C57 Black	"
AKR	"
DBA/2	"

* Six-mo-old Swiss mice of both sexes, and 5- to 6-wk-old male inbred mice were used in this exp. The LD_{50} was defined as No. of organisms/0.5 ml of inoculum given intraper. that killed 50% of test mice in 10 days.

† Experience demonstrated that Todd-Hewitt broth affected results markedly; *freshly* made Todd-Hewitt broth prepared as described by Massell(3) gave consistent results. A study of biochemical components of Todd-Hewitt broth that limit infectivity of group A streptococci for mice is in progress.

TABLE II. Influence of Sex and Age on Susceptibility of Mice to Infection by *Streptococcus pyogenes,* Type 18.

Strain of mice	Sex of mice	Age of mice (mo)	LD_{50}
BALB	♂	6	$<10^{1}$
	♀	6	$10^{3.8}$
	♂ or ♀	1	$<10^{1}$
Swiss	♂	3	$10^{4.9}$
	♀	3	$10^{6.1}$
	♂ and ♀	6	$10^{5.14}$
	" " "	1	$10^{2.56}$
C58	♂	6	$<10^{1}$
	♀	1	$10^{1.5}$
DBA/2	♂ or ♀	1	$<10^{1}$

TABLE III. Infectivity of Group A Streptococci of Diverse Origin for Inbred Male BALB Mice.

Strain of streptococcus	Origin of organism	LD_{50}
Type 1	Freshly isolated from clinical streptococcal septicemia	$10^{5.78}$
1-L	Mouse adapted strain of type 1 organisms*	$10^{2.12}$
4-Ase	Non-encapsulated strain of *Streptococcus pyogenes* known to produce large quantities of hyaluronidase	$>10^{7.0}$
12	Stock laboratory strain	"
18 M	Isolated from abscess of monkey; passed once in Todd-Hewitt broth	$10^{5.9}$
18 P	Substrain of Type 18 M isolated from laboratory worker infected accidentally; passed once in Todd-Hewitt broth	$10^{1.84}$
18	Substrain of Type 18 P passed once through skin of rabbit as described by Watson(5)	$<10^{1}$
18-4397B	Stock laboratory strain	$10^{5.57}$
19-1236	Freshly isolated from patient with streptococcal conjunctivitis; passed once in Todd-Hewitt broth	$>10^{7}$
28	Stock laboratory strain	"

* Kindly sent by Dr. R. Lancefield.

died without remarkable gross pathologic changes. In contrast, adult BALB female mice that survived an LD_{50} dose of organisms provided a consistent pattern of reaction to infection: suppurative lesions formed at the site of inoculation or in the extremities, became necrotic and sloughed. In these mice chronic infection persisted for 1 to 4 months to eventuate in death in most cases. In a small number of cases (1 to 2%) C58 mice that survived infection remained asymptomatic until the third month after inoculation when suppurative lesions occurred in joints and limbs. Organisms recovered from BALB and C58 mice were identified by cultural and serologic tests as *Streptococcus pyogenes,* type 18. Thus, survival of group A streptococci in mice for 3 to 4 months was established. Swiss mice that survived infection, in distinction to BALB and C58 mice, were not ill at any time nor was there suggestive evidence of chronic infection.

Discussion. An analysis of host-parasite relationships is facilitated by the availability of a sensitive assay system for factors which govern the virulence of an organism or determine resistance or susceptibility of the host. The finding(6) that inbred mice were highly susceptible to infection by an unadapted group A streptococcus could be significant since it may contribute toward elucidation of the complex factors involved in the reaction of the host to streptococcal infection. An indication of the nature of these factors was suggested by observation that: a) only a single strain of serotypes of group A streptococci tested was highly virulent to mice; b) sex and age of mice influenced susceptibility to infection; c) the genetic constitution of mice markedly controlled reaction to virulent organisms; and d) latent streptococcal infection persisted for 3 to 4 months in C58 mice. Denny and Thomas(7) reported survival for 104 days of group A streptococci in tissues of rabbits infected experimentally. The finding that group A streptococci could survive for relatively long periods of time in mice lends credence to the view that latent infection may be important in the induction of late sequelae to streptococcal infection.

Summary. Less than 10 organisms of an unadapted strain of *Streptococcus pyogenes,* type 18, caused fulminant infection in BALB, BALB/c, AKR, DBA/2, C58 and C57 Black inbred mice. Pen bred Swiss albino mice were significantly more resistant to infection. Inbred BALB and C58 mice that survived the original inoculum underwent latent or chronic infection; organisms were recoverable from untreated mice for at least 3 to 4 months. Data were obtained which revealed that susceptibility of mice to infection depended on strain of serotype of the organism tested as well as the genetic constitution, age and sex of the host. These studies demonstrated that a spectrum of diseases could be produced by employing a single strain of *Streptococcus pyogenes,* type 18, and different strains of inbred mice. The application of these findings to study of strep-

tococcal infections and their late sequelae was discussed.

1. Rothbard, S., *J. Exp. Med.*, 1948, v88, 325.
2. Committee on Standardized Nomenclature for Inbred Strains of Mice, *Cancer Res.*, 1952, v12, 602.
3. Massell, B. F., *Naval Research Unit No. 4*, 1950, p44.
4. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.
5. Watson, D. W., In: *Streptococcal Infection* (M. McCarty, Editor), Columbia University Press, New York, 1954.
6. Murphy, W. H., and Watson, D. W., *Bact. Proc.*, 1956, 89.
7. Denny, F. W., Jr., and Thomas, L., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 260.

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Developing Blood Brain Barrier to Trypan Blue.* (23077)

FRED M. GRAZER AND CARMINE D. CLEMENTE
(Introduced by D. C. Pease)
Department of Anatomy, School of Medicine, University of California at Los Angeles and Veterans Administration Hospitals, Los Angeles and Sepulveda, Calif.

Ever since Ehrlich first observed that the central nervous system could not be stained by intravenous injections of acid aniline dyes (1), information dealing with the functional and structural embryonic development of the blood brain barrier has been of considerable importance to physiologists. Bakay(2) was able to show by use of radioactive P^{32} that embryonic rabbit brain was more permeable to this ion than adult brain, and that permeability to P^{32} is greatest in early intrauterine life but continues to decrease until about 7 weeks of postnatal development. Grontoft (3) perfused human and animal fetuses with trypan blue and found the brain normally impermeable to this vital dye. Ten-minute delivered human fetuses 5 to 30 cm long had already developed an impermeability to trypan blue. It appears, however, that no one has observed penetration of vital dyes into the embryonic nervous system *in vivo*, due to the permeability problem of placental barrier and the difficulty of injecting embryos directly, although Waddington and Carter(4) were able to induce abnormalities in mouse embryos by injection of trypan blue into pregnant females.

In this study, an attempt was made to show *in vivo* staining characteristics of the rat embryo with trypan blue, and special emphasis was placed on development of blood brain barrier with respect to developing CNS blood vessels. All observations are made *in vivo* with the exception of the youngest rat embryos (10½ days, about 3 mm), which were perfused with trypan blue in Ringer's Solution while the heart was still beating.

Methods. Four-month-old Wistar female rats were used, and exact gestation times were determined by vaginal smears. If insemination had occurred, the female was separated from the male and used for experimentation at the appropriate gestation time. An error of about 12 hours in embryo age is possible with this method. Rat embryos were injected intraperitoneally or via amniotic circulation with a saturated trypan blue solution. Gestation ages in litters studied ranged from 10½ days to birth. Embryos of 10½ and 11 days were perfused with dye after delivery of the fetuses into Ringer's Solution at 37°C since it was extremely difficult to make successful *in vivo* injections in embryos younger than 12 days. Following injection, these fetuses were placed immediately in 10% formalin. All *in vivo* injections were made through the wall of the uterus, which was rendered transparent with proper illumination. Intravascular injections of the amniotic circulation were suc-

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