value has been eliminated.

The procedure as described requires no special equipment other than an ultraviolet spectrophotometer. However, the need for scrupulously clean glassware cannot be overemphasized. An analysis may be completed easily within one working day and many may be run simultaneously. The residues following chloroform removal may be stored and this frequently allows a more flexible operation.

After completion of these studies, the method of Spingler and Kaiser(6) appeared, also based on the intense absorption by Tolbutamide at 228 mμ. Utilizing an extraction of lyophilized serum, these investigators compensated for the contribution of the serum by a second absorbance measurement at 280 mμ and treatment of the system as a 2-component mixture (blank + Tolbutamide). Losses of about 30% of the Tolbutamide present were encountered. The procedure presented herein is simpler, losses encountered are considerably less (about 15% for human plasma and 9% for dog plasma), and special apparatus such as lyophilizing equipment is unnecessary. Spingler and Kaiser(6) report a half-life of 8.7 hours for Tolbutamide in the human. The present studies have indicated a half-life of 13.7-17.6 hours in the dog. Species differences in rate of metabolism of the drug appear to be considerable.

Summary. A procedure is presented for determination of Tolbutamide in plasma. The method consists of a chloroform extraction of weakly acidified plasma, concentration of the extract to dryness, dissolution of the residue in a measured volume of ethanol, treatment of the resulting solution with Darco G-60, and measurement of the absorbance of the resulting charcoal filtrate at 228 mμ. Recoveries of Tolbutamide from human and dog plasmas have been satisfactory and plasma levels determined by this procedure have correlated well with the hypoglycemic response elicited by the drug.

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1. O'Donovan, C. J., *J. Chronic Dis.*, 1956, v4, 635.
2. Johnson, J. L., Struck, W. A., Scott, E. J., and Stafford, J. E., *Anal. Chem.*, 1953, v25, 1490.
3. Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, v41, 367.
4. Bratton, A. C., and Marshall, E. K., Jr., *ibid.*, 1939, v128, 537.
5. Forist, A. A., and Chulski, T., *Metabolism*, 1956, v5, 807.
6. Spingler, H., and Kaiser, F., *Arzneimittel-Forschung*, 1956, v6, 760.

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Increased Susceptibility to Herpes Simplex in Mice Subjected to Avoidance-Learning Stress or Restraint.* (23426)

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A variety of factors other than specific immunity modify host response to infectious agents, protecting against repeated exposure to potentially dangerous agents on the one hand, and subtly converting innocuous exposures and inapparent infections into overt disease on the other(1). Psychologic or emotional stress is often proposed as one of the factors predisposing to overt infection(2). Clinical evidence supporting an etiologic role for emotional stress in recurrent herpes simplex is particularly suggestive(3,4), but experimental data are lacking. As the initial step in a study aimed toward elucidation of psychologically induced changes in resistance, an experimental system is described here

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which demonstrates a stress-induced increase in susceptibility of mice to infection with the virus of herpes simplex.

Materials and Methods. Four to 6-week old mice weighing 15 to 20 g from a colony of a selected strain of Salmonella resistant Swiss mice were employed.† The mice were fed a commercial mouse ration.‡

Stressing Method. Choice of stressor was guided by 4 considerations: (1) desire for a sustained emotional disturbance, (2) minimization of physical stress which might directly influence resistance, (3) maintenance of nutritional status and (4) applicability to large numbers of animals. Effective, widely employed, stress situations utilizing approach-avoidance conflicts around satisfaction of hunger and thirst were not used because of the second and, particularly, the third considerations mentioned.

The first stressing procedure was an avoidance-learning situation utilizing a shuttle box type of stress cage. Dollard and Miller(5), Mowrer(6) and others have demonstrated that with fear produced by painful shock, animals become rapidly conditioned to the buzzer, light signal and cage in which shock is administered.

The avoidance learning paradigm partially satisfies the requirements listed above for a stress situation. The procedure can be repeated at frequent intervals for several hours. The temporal sequence of stimulus presentations can be varied as disturbance levels decline. At the same time, each mouse, by virtue of avoidance responses, receives shock infrequently. The method does not employ nutritional restriction and is adaptable to large numbers.

Stress cages, 10 x 41 x 5 inches deep, with grid flooring, were partitioned with opaque plastic to form 10 compartments of approximately 4 x 10 inches, each housing one mouse. The outer walls and removable cage tops were of clear plastic to permit observation. The smooth plastic surfaces prevented mice from escaping the stress. Each compartment was divided into two 4 x 5 inch sections by plastic barrier rising 1½ inches above grid floor. A low voltage, low amperage A.C. current could be passed through the grid flooring on either side and through parallel wires ⅛ inch above the top of each barrier to discourage barrier sitting. Presentation, duration, and interval between stimuli were controlled by an electronic programming apparatus permitting independent variation of duration and interval between stimuli. The following sequence was utilized: A loud buzzer and a bright light were turned on for 5 seconds. As this conditioned stimulus, or signal for a shock avoiding barrier jump, was terminated, a current, the unconditioned stimulus, was passed through the grid floor on one side. After 5 minutes, the conditioned stimulus was repeated and the current reversed to the opposite side.

Stress procedure I. At the beginning of each experiment, mice were selected so as to equalize litter representation and mean weight and placed in home cages with food and water *ad libitum*. Each experimental day the animals were transferred from home cages and subjected to the stressing procedure for 6 hours. Control animals were placed in identical cages and were stimulated with light and buzzer, but not current. Food and water were withheld from stressed and control animals during the 6 hour experimental period. Animals in preliminary experiments were observed hourly for avoidance responses and for signs of behavioral disturbance. Cumulative observations of avoidance provided an approximation of amount of shock sustained by animals in subsequent experiments. Behavioral observations on selected animals were utilized in ratings of disturbance levels. At the end of 6 hours, all animals were replaced in home cages.

Within 3 to 6 days, stressed animals learned to avoid the electrical shock 50-80% of the time, and disturbance levels tended to decline. The stress situation was then modified occasionally by changing the duration of the stimuli. The number of days of stress ranged

† The strain of mice was a highly inbred strain developed by Dr. Leslie T. Webster of Rockefeller Institute for Medical Research. The original pair was obtained from Dr. Howard A. Schneider in 1941 and the colony was maintained by random mating without further selection or inbreeding.

‡ Purina laboratory chow.

FIG. 1. View of 3 sections of avoidance-learning cage. Photograph was taken a moment after the conditioned stimulus, light and buzzer; the 2 mice on the right have jumped over the barrier and the mouse on the left is preparing to jump to the safe side.

from one to 28 in different experiments. For periods longer than one week, animals were stressed daily for 6 days and rested on Sundays. Stress was terminated at the time of inoculation with virus, and all animals remained in home cages thereafter.

Stress procedure II. The use of the avoidance-learning stress does not completely avoid the possible direct effects of mild electrical shock and increased physical activity. Electrical injury appears unlikely because of the low amperage employed and the activity of stressed animals did not appear to be significantly different from that of controls. However, the 2 possibilities were studied further by use of restraining stress procedure adapted from that employed by Hamilton for rats(7) and by Bartlett, *et. al.*, for mice(8) which eliminated electrical stimulation and minimized physical activity. Experimental animals were loosely wrapped for 6 hours in fine mesh copper screen which produced effective restraint (Fig. 2). Control animals were maintained in home cages from which food and water were removed for the same period.

Experimental infections. The HF strain of the virus of herpes simplex was used. Stocks of virus were prepared in the form of 10% suspensions of pooled infected mouse brain in distilled water and preserved in sealed tubes in a dry ice chest. Animals were inoculated at the desired time by the intraperitoneal injection of 0.1 ml of diluted virus containing 100 LD_{50} as determined by conventional intracerebral inoculation. Virus so administered usually resulted in fatal infection in 50 to 60% of control mice within 14 days. Survivors ordinarily resisted intracerebral challenge, indicating that mice which did not develop overt disease had become immune. The amount of viral antigen in the inoculum was far too small to stimulate significant active artificial immunity; resistance to challenge is, then, evidence of inapparent infection. Animals were observed daily. Variations in the course of the disease in the different groups were manifest as differences in incubation period, in survival time, or in the incidence of mortality. The most apparent differences between experimental and control groups were in survival time and mortality. Survival times and mortality rates were, therefore, employed in evaluating the effect of the experimental procedures.

Results. Avoidance-learning stress. In a representative experiment, Exp. 22, 175 male and female mice averaging 18 g in weight were divided into 4 groups. Group A received avoidance-learning stress 6 hours per day for 28 days. Beginning with the 15th experimental day and continuing for 14 days thereafter, Group B was subjected to the same stress for 6 hours per day. On the 28th experimental day, Group C received 6 hours of stress. Animals in Group D, the control group, were placed in cages of the same type for 6 hours per day throughout the 28 day stress period to equalize the handling factor. On the 29th day, all animals were inoculated with virus. The results of this experiment,

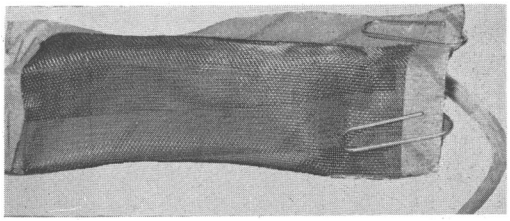

FIG. 2. Mouse restrained in copper screen. Rough edges are covered with masking tape. The front is closed permanently with wire staples; the rear edges are free to introduce and remove the mouse. After the mouse is in place the free edges are overlapped and clamped with paper clips.

TABLE I. Total Deaths and Death Rates in Animals Stressed by Avoidance Learning and Infected with Virus of Herpes Simplex.*

Group	N	Daily deaths: Days post-inoculation 6	7	8	9	10	11	12	13	14	15	16	Deaths over total N	Total deaths: Groups compared	X^2	Signif. level
A. Pre-inoc. stress 28 day	42	0	6	12	2	3	2	4	2	0	0	0	31/42	A by D	7.73	.005
														A " B	2.63	
B. 14 "	45	2	2	4	6	3	3	3	2	0	0	0	25/45	A " C	8.88	.005
C. 1 "	43	0	2	8	1	2	1	3	1	0	0	0	18/43	B " C	1.65	
														B " D	1.11	
D. Control	45	0	2	3	4	4	1	3	3	0	0	0	20/45	C " D	.06	

* 0.1 ml iper. containing 100 intracer. LD_{50}.

summarized in Table I, suggest a positive relationship between length of the stress and mortality in each group. Chi-square tests for the differences among groups in mortality are recorded in the last 3 columns. Significant differences were found between 28 day stress and control groups, and 28 day and one day stress groups. The number of deaths in the 14 day stress group exceeded that in the control and one day stress groups although the latter differences were not statistically significant for the sample sizes utilized. Groups were also compared for mean and median survival times. An inverse relationship between duration of stress and survival time was suggested, but was not statistically significant.

Confinement stress. No correlation was found in the experiment described in Table I between either mortality or survival period and the amount of shock encountered. In view of the crudeness of the measure, the results could have been due to shock, *per se,* or, possibly, to increased activity. To test these questions, the experiment was repeated, substituting the confinement procedure described, for the avoidance-learning stress. The results summarized in Table II were similar to those obtained with avoidance-learning, although the differences obtained between death rates are smaller, and fewer animals in all groups died. Death rate comparisons again yielded a significant difference between 28 day stress and control animals. No other differences between groups are significant, but the trend of association between mortality and amount of stress is similar to that found in the previous experiment. Tests of survival time did not yield significant differences between groups. However, combined survival time data for the 28 and 14 days stress groups, when compared with similar data for the control and one day stress groups, indicate a trend which is similar to that with avoidance-learning stress. Animals subjected to longer periods of stress tended to succumb sooner than minimally stressed or control animals.

Influence of sex. The experiments presented in Tables I and II suggested a sex difference in resistance to brief, as contrasted to

TABLE II. Total Deaths and Death Rates in Animals Stressed by Confinement and Inoculated with Herpes Simplex.*

Group	N	Daily deaths: Days post-inoculation 6	7	8	9	10	11	12	13	14	15	16	Deaths over total N	Total deaths: Groups compared	X^2	Signif. level
A. Pre-inoc. stress 28 day	40	0	2	5	3	3	4	4	2	0	0	2	25/40	A by D	3.92	.05
														A " B	1.21	
B. 14 "	37	1	1	7	3	2	1	2	0	1	0	1	19/37	A " C	3.25	
C. 1 "	38	0	2	2	1	2	4	2	1	2	0	0	16/38	B " C	.64	
														B " D	.99	
D. Control	40	1	3	2	2	2	0	3	2	0	0	1	16/40	C " D	.03	

* 0.1 ml iper. containing 100 intracer. LD_{50}.

TABLE III. Total Deaths and Death Rates in Male and Female Mice Stressed by Avoidance-Learning and Inoculated with the Virus of Herpes Simplex.*

Group	N	Daily deaths: Days post-inoculation 6	7	8	9	10	11	12	13	14	15	16	Deaths over total N	Total deaths: Groups compared	X^2	Signif. level
A. ♀, 14 day pre-inoc. stress	20	0	4	3	3	2	0	4	1	0	0	0	17/20	A by D	5.58	.02
B. ♀, 7 day pre-inoc. stress	20	1	4	4	4	0	0	2	1	0	0	0	16/20	A " B	.173	
C. ♀, exp. cage control	20	0	1	2	1	2	0	2	2	1	0	0	11/20	A " C	4.28	.05
D. ♀, home cage control	20	0	3	2	0	2	1	2	0	0	0	0	10/20	B " C	2.56	
E. ♂, 14 day pre-inoc. stress	19	2	4	1	0	1	1	2	0	2	0	1	14/19	A " E	.765	
F. ♂, home cage control	20	0	1	1	1	1	0	1	1	1	0	0	7/20	D " F	.920	
														E " F	4.91	.05

* 0.1 ml iper. containing 100 intracer. LD_{50}.

longer stress periods. Table III summarizes an experiment designed to test those factors. Eighty females were divided into 4 groups equalized for mean weight and litter representation. As recorded in Table IV, Group A was subjected to 14 days of avoidance learning stress, and Group B to 7 days of stress. Group C animals were placed for 6 hours per day in experiment cages through which no shock was passed, while Group D remained in home cages from which food and water were removed during the 6 hour stress period each day. Fourteen day stress (Group E) and home cage control male groups were also used. Comparison of total deaths in the various groups given in Table III indicates significant differences between females stressed for 14 days and both experimental and home cage controls. More animals died following inoculation in the female group previously stressed for 14 days than in either of the female control groups. A similar significant difference was found between male 14 day stress and control groups. Other differences between conditions, sex comparisons included, did not approach the .05 level of significance. The proportion of females dying in both experimental and control conditions was higher than that found in males. Again, the survival time in both male and female groups subjected to stress tended to be shorter than the survival period for control animals. The difference between the 14 day stress and control groups in the females is somewhat greater than that seen

TABLE IV. Chi Square Test* of Relation between Weight Gain and Death Rates in Experiments Summarized in Tables I, II, and III.

Exp.	Within groups: Group	X^2	P level	Combined groups: Group	X^2	P level
E 22	4 wk stress	1.6		4 wk control	9.5	.003
	2 " "	1.2		2 " "	.03	
	1 day "	4.8	.03	4 wk-2 wk	2.9	.09
	Control	1.1		1 day control	1.1	
				ALL GROUPS	8.8	.003
23	4 wk stress	1.06		4 wk control	4.05	.05
	2 " "	.02		4 wk-2 wk	.62	
	1 day "	1.72		2 wk control	1.71	
	Control	.07		1 day control	.51	
				ALL GROUPS	.05	
26	2 wk stress	.50		2 wk control	1.3	
	1 " "	.01		1 " exp. cont.	7.2	.008
	Exp. cont.	.04				
	Cage cont.	.90				

* Fourfold tables, D.F. = 1.

in earlier studies utilizing combined male and female samples.

Influence of stress on growth and of growth on susceptibility. By withholding food and water from both stressed and control animals during the 6 hour stress periods, and providing food and water *ad libitum* for the balance of each day, it was hoped that differences in growth between control and stressed animals would be minimal. However, inspection of the weights of the animals in Exp. 22 and Exp. 23 revealed that the stressed animals did not gain weight at the same rate as did control animals. For example, mean initial weight of the group subjected to avoidance-learning stress for 4 weeks was 20.28 g and mean final weight at the termination of stress was 23.0, a gain of 2.72. Weights of the comparable controls were 20.4 and 25.8 with a gain of 5.4. Similarly, the mean gain for the 4 week confinement stress group in Exp. 23 was 2.17 while the gain for the controls was 5.55.

Was the failure to gain weight directly related to and, possibly, the cause of the observed increase in susceptibility, or was the partial suppression of growth an independent response to the stress situations? Further inspection of growth records revealed considerable variation in response of individual animals. For example, some stressed mice gained at essentially the same rate as did controls while others gained much less rapidly. If increased susceptibility were caused by nutritional deficit and resulting failure to gain weight, a correlation between weight change and susceptibility could be expected within groups of animals subjected to the same experimental conditions. Data from the experiments reported here were examined for this possibility, using chi square to test for association between weight change and death or survivals in each experimental and control condition. The results are summarized in Table IV. As anticipated, the correlation between failure to gain weight at the normal rate and susceptibility is striking when experimental and control groups are combined. On the other hand, of 12 comparisons within groups, only one yielded a chi square significant beyond the .05 level. In most instances the chi squares were negligible and no trend towards positive or negative association was apparent. Thus, when the stress factor was held constant, there was no consistent relationship between susceptibility and weight change and failure to gain rapidly could not be directly related to increased susceptibility.

Discussion. A stress-induced increase in susceptibility to infection with the virus of herpes simplex in mice, manifested by increase in death rates and a trend toward decreased survival times in stressed as compared with control groups was demonstrated. Whether or not our experimental stress is analogous to emotional or psychological stress in man is questionable. However, the stresses resulted in behavioral changes usually associated with emotional disturbance in rodents, *e.g.*, tremor, crouching, defecation, exaggerated startle reaction, and vocalization.

The similarity of response to 2 different forms of stress; the one, fear of electrical shock, in which a possibility of associated electrical injury and increased physical activity could not be completely excluded, and the other, forced restraint, in which direct trauma and physical activity were avoided, suggest a common, centrally mediated response. Additional experimental evidence supporting a relationship between higher nervous centers and susceptibility has been reported by Tobach and Bloch(9) who observed correlations between behavior and susceptibility to tuberculosis in mice and rats and by Teodoru and Schwartzman(10) who have found that a variety of factors influence the susceptibility to poliomyelitis in hamsters. By showing that an environmental situation as routine as that obtaining in daily examination of experimental animals can increase susceptibility to poliovirus, the latter authors emphasize the extreme sensitivity of the balance between host and parasite in their experimental system.

Failure to gain weight as rapidly among stressed groups as among controls, together with the observations of Schaedler and Dubos (11) that mice starved for 24 hours exhibit an increased susceptibility to bacterial infection suggest a direct relation between impaired nutrition and the increased susceptibility observed in our experiments. Conversely, when the response of individual animals was ex-

amined, there was no correlation between rate of growth and susceptibility or resistance, a result in accord with experience summarized by Clark, McClung, Pinkerton, Price and Schneider indicating that animals will tolerate severe restrictions of food over long periods of time without significant changes in susceptibility providing specific deficiencies of essential nutrients are excluded(12). In comparing the experiments reviewed by Clark, *et. al.* (12) and the recent work of Schaedler and Dubos(11), the difference in partial deprivation of food or nutrients and complete starvation must be kept in mind. Absence of direct correlation between failure to gain and susceptibility in our experiments indicates that nutritional impairment and increased susceptibility to herpes simplex were independent responses to the experimental stress.

Summary. Stress induced in mice by avoidance-learning or by restraint resulted in an increased susceptibility to infection with the virus of herpes simplex.

1. Dubos, R. J., *Biochemical Determinants of Microbial Diseases,* Harvard Univ. Press, Cambridge. 1954, Chap. 1.

2. Wolfe, H. G., Charles C. Thomas, Springfield, Ill., 1953, 199.

3. Dunbar, H. F., Columbia Univ. Press, N. Y., 1938, 2nd ed., 832.

4. Blank, H., and Brody, M. W., *Psychosomatic Med.,* 1950, v12, 255.

5. Dollard, J., and Miller, N. E., *Personality and Psychotherapy,* McGraw-Hill Book Co., N. Y., 1950.

6. Mowrer, O. H., *Learning Theory and Personality Dynamics,* Ronald Press Co., N. Y., 1950.

7. Hamilton, J. B., *J. Lab. Clin. Med.,* 1937, v22, 466.

8. Bartlett, R. G., Hemeldach, R. H., and Bohr, V. C., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 4.

9. Tobach, E., and Bloch, H., *Adv. Tuberc. Res.,* 1955, v6, 62.

10. Teodoru, C. V., and Schwartzman, G., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 181.

11. Schaedler, R. W., and Dubos, R. J., *J. Exp. Med.,* 1956, v104, 67.

12. Clark, P. F., McClung, L. F., Pinkerton, H., Price, W. H., and Schneider, H. A., *Bact. Rev.,* 1949, v13, 99.

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Production of Hypertonic Urine in Humans in the Probable Absence of Antidiuretic Hormone (ADH). (23427)

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Shannon observed the formation of concentrated urines in severely dehydrated dogs with experimental diabetes insipidus(1). Subsequently, Homer Smith and his associates(2) suggested the presence of an "ultimate" concentrating segment (possibly the collecting tubule) where, independently of ADH, water was continuously reabsorbed without solute. In this conceptual scheme, the primary function of ADH is to allow the back diffusion of water when solute is actively reabsorbed in the "penultimate" portion of the distal tubule, permitting delivery of a small volume of isosmotic fluid to the final segment, where a hypertonic urine may be formed.

Recent animal experiments suggested that this does occur and that ADH is not essential for production of a hypertonic urine(3,4). Berliner and Davidson(3) produced "physiologic diabetes insipidus" in dogs by sustained water loading and simultaneously collected urine from each kidney. When the glomerular filtration rate was considerably reduced on one side by compression of the renal artery, urinary flow and solute excretion fell markedly while urinary solute concentration increased to a level greater than that of the plasma. Urine from the contralateral side remained hypotonic, indicating that the experimental procedure did not cause the liberation