sive shocks appears to make it inadvisable to give electroconvulsive treatment to patients taking benactyzine.

Summary. Benactyzine (β-diethylaminoethyl benzilate hydrochloride) an antispasmodic that has been used in the treatment of psychoneuroses causes hyperexcitability and clonic convulsions in animals. A taming effect was not observed. Hexobarbital anesthesia was markedly prolonged, the effects of acetylcholine on the isolated gut and blood pressure diminished and the action of epinephrine on blood pressure enhanced by this compound. Spinal reflexes were not affected and cortical and subcortical electrical activity was slowed. Mice receiving benactyzine often died during electroshock seizures which were tolerated by normal animals.

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The Development *in vitro* of an Avirulent, Immunogenic Variant of *Mycobacterium tuberculosis* var. *hominis*.* (22546)

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Youmans and Youmans(1) reported that the virulent H37Rv strain of *Mycobacterium tuberculosis* var. *hominis* would multiply in a modified synthetic Proskauer and Beck medium from which the nitrogen source, asparagin, had been omitted, at a rate similar to that obtained in the complete medium. During the course of subsequent investigations, the H37Rv strain was subcultured serially once a week for approximately 6 years onto the surface of the Proskauer and Beck medium from which the nitrogen source had been omitted. It was found that the virulence of these cultures, when tested at intervals in mice and in guinea pigs, had decreased markedly. This variant of the parent H37Rv strain has been designated H37RaN, which stands for strain H37, rough, avirulent, nitrogen.

This study is concerned with a) the virulence of H37RaN as compared with the parent H37Rv, b) metabolic differences between the 2 strains, and c) the immunogenic activity of H37RaN as compared with the well known immunogenic mycobacterial strains, H37Ra and BCG.

Methods. The strains of mycobacteria employed were the 3 human strains, H37RaN, H37Rv, H37Ra, and the one bovine strain, BCG. The H37Rv and H37Ra strains were maintained as pellicle cultures on the modified synthetic Proskauer and Beck (P & B)

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medium(2) by weekly subculture. The variant, H37RaN, was grown in a similar fashion on the P & B medium except that the nitrogen source, asparagin, was omitted. Because of the slight filmy growth on this medium, it was necessary to make one subculture onto the complete P & B medium in order to have sufficient cells to make a suspension for experimental work. Cells of the BCG strain were kindly supplied by Dr. Sol Rosenthal of the Tice Clinic of Chicago at the time of each experiment.

Quantitative growth studies were done by employing the small inocula technic(3). This method consisted of inoculating a set of culture tubes with each of several inocula which varied in size from 10^{-1} mg through 10^{-6} mg, wet weight. The time in days when growth first occurred for each inoculum was recorded, and this figure was plotted against the logarithm of the inoculum size. From the straight line so obtained the rate of growth and generation time of the organisms were determined.

The basal medium employed was the modified P & B medium from which the carbon source, glycerol, was omitted. Dilutions of the carbon substrates related to the tricarboxylic acid cycle were made with the basal medium. The media were sterilized by sintered glass filtration and tubed aseptically. After inoculation, the cultures were incubated at 37°C and examined daily for the presence of growth. At least 2 tubes of each medium were used for each inoculum and all experiments were repeated 2 to 4 times.

In the animal experiments 3 procedures were used: a) The virulence of the H37RaN and the H37Rv strains was tested by injecting intravenously groups of 10 to 20 Strong A mice, 18-20 g in weight, with 1.0, 2.0 or 4.0 mg, wet weight, of a standardized ground suspension prepared from mature pellicles (3) grown on the complete P & B medium. The time when each mouse died and the autopsy findings were recorded. Following the method of Litchfield(4) the time of death, in days, was plotted against the cumulative percent mortality on logarithmic probability paper and from the straight line so obtained, the median survival time (ST_{50}) was determined, and the 95% confidence limits were calculated.

b) The virulence of H37RaN was tested in three 300 g guinea pigs by injecting 0.1 mg, wet weight, of the standardized ground suspension intraperitoneally. At the end of 4 months, the guinea pigs were killed, autopsied, and the organs examined macroscopically for tuberculous lesions.

c) The immunogenic activity of H37RaN, BCG and H37Ra was examined in mice by injecting intraperitoneally 1.0 mg, wet weight of standardized ground suspensions of live and heat killed cells. The heat killed cells were autoclaved at 126°C for 15 minutes. The mice were challenged 4 weeks later by infecting with 1.0 mg of H37Rv intravenously. In the evaluation of the results of these experiments, a different procedure was used. Immune mice die over a prolonged period after challenge and, consequently, the normal distribution curve on which the median survival time calculation is based is not obtained. Therefore, the percent of mice which lived longer than 29 days was used as an index of the degree of immunity, since approximately 95% or more of the controls die before this time.

Results. Morphology and cultural: H37RaN grew as a slight, filmy pellicle on the surface of the nitrogen-free P & B medium and resembled macroscopically the very young pellicle cultures of the virulent parent H37Rv strain. Cording was noted, and microscopically, the organisms were acid-fast and in all respects similar in appearance to the organisms of the parent H37Rv strain. On the complete P & B medium the H37RaN strain again resembled the growth of the H37Rv strain. The pellicle growth was luxuriant and did not contain "rough" patches. It did not resemble the heavy waxy pellicle growth produced by the avirulent H37Ra strain.

Virulence. The virulence of H37RaN was tested first in mice after one year of continual subculturing on the nitrogen-free P & B medium. The median survival time of the mice infected intravenously with 1.0 mg of H37RaN was 22.5 days, and the median survival time of the control mice infected with

TABLE I. Effect of Compounds Related to the Tricarboxylic Acid Cycle on Growth of Strains H37RaN and H37Rv of *M. tuberculosis* var. *hominis*.

Substrate	Concentration of substrate in %								
	1.0		0.25		0.06		0.016		0
Strains:	aN*	Rv†	aN	Rv	aN	Rv	aN	Rv	
Glycerol	26.4‡	21.7	10^{-3}§	21.7	10^{-3}	24.0	10^{-3}	24.0	
Lactic acid	10^{-2}	0	10^{-1}	22.7	10^{-2}	22.7	10^{-2}	22.7	
Pyruvic "	10^{-3}	0	24.0	20.5	10^{-2}	20.5		21.7	
Acetic "	0	0	10^{-2}	10^{-2}	10^{-1}	25.2	10^{-3}	25.2	
Citric "	0	0	0	0	0	0	0	0	
Oxalosuccinic acid			10^{-1}	0	28.8	10^{-2}	10^{-1}	25.2	
α-Ketoglutaric "	0	21.7	0	21.7	0	21.7	0	21.7	
Succinic acid	0	0	0	0	0	0	0	0	
Fumaric "	0	0	0	0	0	0	0	0	
Malic "	0	0	0	0	0	0	0	0	
Oxaloacetic acid		0	10^{-1}	21.7	10^{-1}	21.7	0	21.7	
DL-Alanine	0	0	0	0	0	0	0	0	
Glutamic acid	0	0	0	0	0	0	0	0	
Aspartic "	0	0	0	0	0	0	0	0	
None									0

* Strain H37RaN. † Strain H37Rv. ‡ Generation time in hr. § Smallest inoculum (in mg) which grew.

1.0 mg of the parent H37Rv strain was 14.5 days. The virulence of this strain was tested again after 3 years of continual subculturing on the nitrogen-free medium. The mice, which were infected with 1.0 mg of these organisms intravenously, were sacrificed 4 months after injection, and on autopsy revealed no macroscopic tuberculous lesions. Further experiments were done in which the amount of H37RaN injected intravenously into the mice was increased to 2.0 and to 4.0 mg. These mice were killed after 4 months and on autopsy no gross tuberculous lesions were seen. In comparison to these findings, mice infected with 2.0 mg and 4.0 mg of the parent virulent H37Rv strain had median survival times of 9.5 and 3.5 days, respectively. In the following 3 years, this strain has been subcultured on the same nitrogen-free medium, and the virulence has been tested several times in mice employing a 4.0 mg inoculum. None of the mice showed any evidence of tuberculosis, even those sacrificed as long as 8 months after injection. The virulence of this organism was tested in guinea pigs after the 3 year period and also appeared to be non-virulent for these animals. The animals were sacrificed 16 weeks after injection, and on autopsy found to have no gross tuberculous lesions. The H37RaN strain was subcultured serially at weekly intervals on the complete P & B medium to determine whether virulence would be restored by the addition of the nitrogen source, asparagin. The last such subculture to be tested for virulence in mice was the 36th. Four of the 10 mice injected with 3.0 mg died between the 2nd and 7th months of tuberculosis and the remainder were sacrificed 7 months after infection. Upon autopsy, the lungs of these mice were grossly tuberculous with about 25-50% of the lung tissue involved.

Metabolic studies. The results of these studies are shown in Table I. If growth occurred with several dilutions of the inocula, thus permitting the rate of growth to be determined, the generation time in hours is recorded; otherwise, either the smallest inoculum which grew, or no growth, is recorded. The results with the H37Rv strain given in the table were the same as those reported previously(5). A generation time for H37RaN could be determined for only one concentration of glycerol, pyruvic and oxalosuccinic acids. The growth of only the larger inocula of H37RaN was supported by lactic, acetic, oxaloacetic acids. Alpha-ketoglutaric acid did not support the growth of any inoculum. The parent H37Rv strain grew well in several concentrations of each of these substrates. No growth occurred with either strain in the presence of citric, isocitric, *cis*-aconitic, succinic, fumaric, malic, as-

TABLE II. Response of Immunized Mice to Infection with *Mycobacterium tuberculosis* var. *hominis* (H37Rv).

Vaccine inj. (1 mg)	No. of mice*	% lived >29 days
H37RaN	35	82.9
H37RaN (heat killed)	20	50.0
H37Ra	40	77.5
H37Ra (" ")	39	35.9
H37Rv (" ")	38	34.2
BCG	68	82.3
BCG (" ")	71	31.0

Less than 10% of the control mice lived longer than 29 days.

* Mice which died before challenge are not included.

partic, glutamic acids and alanine.

Immunogenic studies. The results of these experiments are found in Table II. Live cells of H37RaN, H37Ra, and BCG strains immunize to a similar degree, as do the killed cells of H37RaN, H37Ra, BCG and H37Rv; however, killing the cells with heat significantly reduced the immunogenic activity of the strains. The values given in the table which indicate the number of mice which lived longer than 29 days are somewhat high. In other experiments in this laboratory, these strains have produced a lesser degree of immunity.

Summary. A variant of the H37Rv strain of *Mycobacterium tuberculosis* var. *hominis* which appears to be avirulent for mice and guinea pigs, was developed *in vitro* by continual subculture over 3 years on a nitrogen-free synthetic medium. This new strain, designated H37RaN, resembled the parent H37Rv strain both macroscopically and microscopically, but differed metabolically from the parent strain in its reduced ability to utilize for growth carbon compounds related to the tricarboxylic acid cycle. This strain immunized mice as well as the frequently used immunogenic strains, H37Ra and BCG.

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Notes on Impeded Estrogens. (22547)

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Huggins and Jensen(1) have recently discussed a category of estrogenic substances which they have called "impeded estrogens," and defined as compounds producing dose-response curves with shallow slopes when assayed on uterine growth. The prototype of this group of substances is estriol. All of the impeded estrogens studied by Huggins and Jensen possessed either a ketone group at position 6 or a hydroxyl group at 16 leading them to conclude that an oxygenated group at 6 or 16 is necessary for impeded status. They also stated that 16-oxoestrone (16-ketoestrone) did not cause an impeded uterine growth response. Certain of these substances have been shown to have the property of inhibiting the uterine stimulating action of estrone when administered simultaneously with estrone(1,2). Observations made in this laboratory confirmed Huggins and Jensen only in part and suggested that extensions of their conclusions are warranted.

Materials and methods. The assay procedure of Rubin *et al.*(3) has been followed. Mice 23-25 days of age were used; the total dose of test material was administered in 3 divided doses in 0.1 ml corn oil each. The injections were given daily for 3 days and