Three of the mice given testosterone propionate had subcutaneous fibrosarcomas and but one occurred in mice of each of the other 2 groups.

Summary and conclusion. Irradiated mice given estradiol benzoate did not have ovarian tumors whereas mice given testosterone propionate (1.25 mg weekly) or sesame oil did. Irradiated mice given testosterone propionate had fewer lymphomas than did the mice given estradiol benzoate or sesame oil.

Received August 29, 1950. P.S.E.B.M., 1950, v75.

Comparative *in Vitro* Response of Tubercle Bacillus (H37RV) to Dihydrostreptomycin, Para-aminosalicylic Acid, and Dihydrostreptomycin-para-aminosalicylate.* (18223)

K. VENNESLAND, J. ALTONA AND R. G. BLOCH.

From the Frank Billings Medical Clinic, Department of Medicine, University of Chicago.

The authors reported results of *in vitro* experiments(1) indicating that the two drugs streptomycin and PAS, when used together, were more effective in inhibiting the growth of tubercle bacilli than either drug used alone. This was also found to be true in *in vivo* studies(2). Hobby(3), studying a PAS salt of streptomycin consisting of 56 parts of streptomycin and 44 parts of PAS, reported that this compound was twice as effective as an equivalent amount of streptomycin used alone. She further reported early studies indicating that strains of tubercle bacilli resistant to the compound did not readily emerge, none having been isolated after a three-month period of trial. This is in sharp contrast to the well known fact that streptomycin-resistant organisms emerge quite rapidly both *in vitro* and *in vivo* when exposed to this drug, but in keeping with reported *in vitro* studies indicating that the presence of PAS together with streptomycin prevents the emergence of resistance(4). PAS-resistant strains of tubercle bacilli have been reported in patients who have received prolonged therapy(5). *In vitro,* however, such resistant strains emerge only after many months of exposure to PAS(6). Attempts to isolate either streptomycin-resistant or PAS-resistant strains from cultures repeatedly exposed to the two drugs used together have been unsuccessful.

The present report deals with a comparative study of the *in vitro* effects of dihydrostreptomycin-para-aminosalicylate, of dihydrostreptomycin and para-aminosalicylic acid used together in amounts equivalent to those present in dihydrostreptomycin-para-aminosalicylate and of para-aminosalicylic acid and dihydrostreptomycin, each used alone, both with regard to the *in vitro* growth inhibiting effect and to the development of resistance.

Method. The dihydrostreptomycin-para-aminosalicylate (DHSM-PAS) used was a chemical compound in which one mole of dihydrostreptomycin (DHSM) was combined with three moles of para-aminosalicylic acid (PAS). DHSM and PAS were used in a 1:3 molecular ratio in experiments in which the 2 drugs were used together. For convenience in this paper, the use of the 2 free drugs together will be designated by the symbol

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

1. Vennesland, K., Ebert, R. H., and Bloch, R. G., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 250.

2. Bloch, R. G., Vennesland, K., Ebert, R. H., and Gomori, G., *Am. Rev. Tuberc.,* 1949, v59, 554.

3. Hobby, G., Regna, P., and Levert, T., *Am. Rev. Tuberc.,* 1949, v60, 808.

4. Graessle, O. E., and Pietrowski, J. J., *J. Bact.,* 1949, v57, 459.

5. Delaude, A., Karlson, A. G., Carr, D. T., Feldman, W. H., and Pfeutze, K. H., *Proc. Staff Meet. Mayo Clin.,* 1949, v24, 341.

6. Steenken, Jr., W., personal communication.

TABLE I. Growth of H37Rv in Dubos Tween-albumin Medium Containing Various Concentrations of DHSM,* DHSM-PAS,† and the Combination of DHSM and PAS.‡

Drug used	DHSM in μg/cc							
	.82	.41	.205	.103	.05	.025	.013	Control
DHSM	0	2+	3+	4+	4+	4+	4+	4+
DHSM-PAS	0	0	2+	3+	3+	4+	4+	4+
DHSM + PAS	0	0	0	+	4+	4+	4+	4+

* DHSM = Dihydrostreptomycin.
† DHSM-PAS = Dihydrostreptomycin-para-aminosalicylate.
‡ PAS = Para-aminosalicylic acid.
The concentrations of DHSM-PAS and the combination of DHSM + PAS is given in terms of the DHSM content.

DHSM+PAS as opposed to DHSM-PAS which refers to the chemical compound. The virulent human strain of tubercle bacilli H37Rv was used as the test organism in the sensitivity tests and as the parent strain in attempts to isolate drug-resistant organisms. In the latter attempts, organisms were serially transferred into increasing concentrations of the drugs. The tube used for each successive transfer was that containing the highest concentration of drug still allowing growth. All sensitivity tests were performed in Dubos Tween-albumin medium to which the drugs were added aseptically. The experiments were set up in triplicate and the final results recorded are the average of the three tubes.

Results. The first studies were made to compare the *in vitro* effectiveness of DHSM, PAS, DHSM-PAS and DHSM+PAS. Table I shows the results of a typical experiment. It will be seen that the inhibiting concentration of DHSM was 0.82 μg/cc; the inhibiting concentration of DHSM-PAS was one containing 0.41 μg DHSM/cc while 0.205 μg DHSM/cc inhibited growth completely when PAS was added in a 1:3 DHSM:PAS molecular ratio. This relationship between the end points of DHSM alone, DHSM-PAS and DHSM+PAS was consistent in all experiments, though the specific end point itself varied slightly. The results would seem to indicate that while the compound (DHSM-PAS) was more effective than an equivalent amount of DHSM, the two drugs DHSM and PAS used together (DHSM+PAS) in concentrations theoretically present in DHSM-PAS were more effective than an equivalent amount of the compound itself. The full significance of this finding remains to be determined.

Results of studies on the emergence of tubercle bacilli resistant to DHSM and to DHSM-PAS are shown in Fig. 1. The fifth transfer of the organisms into DHSM containing Dubos medium resulted in a strain growing readily in 2048 μg DHSM/cc. In contrast, the fifth transfer of organisms into DHSM-PAS resulted in a strain growing readily in only 4.0 μg DHSM-PAS/cc. It was not until the tenth transfer that a strain was obtained which grew readily in 1000 μg DHSM-PAS/cc. When this DHSM-PAS-resistant strain was tested for its sensitivity to DHSM alone, to PAS alone and to DHSM and PAS used together (DHSM+PAS) it was found that they were resistant not only to DHSM-PAS but also to DHSM and PAS whether used alone or together. Organisms which grew readily in 1000 μg DHSM-PAS/cc were not inhibited by 2000 μg DHSM/cc, by 1000 μg PAS/cc or by 1120 μg DHSM/cc plus 880 μg PAS/cc.

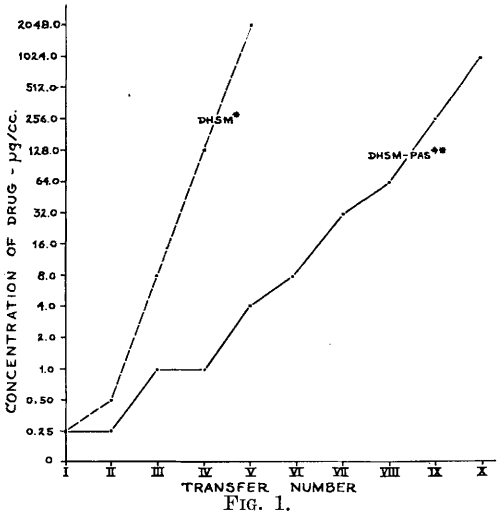

FIG. 1.

Emergence of resistance of H37Rv to DHSM and to DHSM-PAS as the result of serial transfers into increasing concentrations of each drug.
* DHSM = dihydrostreptomycin.
** DHSM-PAS = dihydrostreptomycin-para-aminosalicylate.

No increase in resistance has as yet been demonstrated by exposure of organisms to increasing concentrations of DHSM and PAS used together. These experiments are being continued.

Discussion. While it is recognized that *in vitro* results cannot necessarily be interpreted as *in vivo* effects, the *in vitro* difference between the results when a chemical compound (DHSM-PAS) was used as opposed to when the 2 drugs were added separately, make careful *in vivo* studies important. It had been hoped that the small amount of PAS which could be introduced into the streptomycin molecule might be sufficient to increase the effectiveness of the latter, as well as to reduce the important drawback of the rapid emergence of resistance. These experiments indicate that drug resistant organisms do emerge readily when a strain of tubercle bacilli is exposed to such a compound and that these organisms are resistant not only to the compound itself, but to both streptomycin and PAS as well.

Summary. 1. The *in vitro* growth of the H37Rv strain of tubercle bacilli was inhibited by 0.82 μg DHSM/cc, by a concentration of DHSM-PAS containing 0.41 μg DHSM/cc and by 0.205 μg DHSM/cc when PAS was added in a 3:1 molecular ratio of PAS:DHSM. 2. The fifth serial transfer of H37Rv into increasing concentrations of DHSM resulted in a strain growing readily in over 2000 μg DHSM/cc while the tenth such transfer of the organisms into increasing concentrations of DHSM-PAS resulted in a strain growing readily in over 1000 μg DHSM-PAS/cc. No increase in resistance on the part of organisms transferred into increasing concentrations of DHSM with PAS present in a 1:3 molecular ratio has so far been found. 3. Organisms resistant to the compound (DHSM-PAS) were found to be also resistant to DHSM alone, to PAS alone, and to the mixture of DHSM and PAS.

We are indebted to Mr. William Steenken, Jr., for the H37RV strain of tubercle bacilli and to the Ames Company for their generosity in supplying the dihydrostreptomycin para-aminosalicylate and the PAS used in these experiments.

Received August 29, 1950. P.S.E.B.M., 1950, v75.

Genetic Aspects of the Added Carbon Dioxide Requirements of *Brucella abortus.** (18224)

A. G. MARR AND J. B. WILSON.

From the Department of Agricultural Bacteriology, University of Wisconsin.

It is well known that many strains of *Brucella abortus* require approximately 3 to 10% CO_2 in air for growth on primary isolation(1). However, some isolates lose the requirement for added CO_2 on continued subculture and may then be grown in air alone. The stability of the added CO_2 requirement of 9 strains of *B. abortus* was investigated as an initial step in the study of the role of CO_2 in the metabolism of members of this species.

Methods and cultures. Albimi brucella broth and agar were used as routine culture media and 1 ppm crystal violet was added to the agar for use in making plate counts. The diluent was an aqueous solution of 0.2% Difco tryptose-0.5% NaCl. CO_2 incubations were carried out in vacuum desiccators and the desired pCO_2 obtained by partial evacuation and replacement with tank CO_2. All incubations were at 38°C. Total counts were made using a Quebec colony counter and mutant counts were made using a dissecting microscope with transmitted light. Cultures

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

1. Wilson, G. S., *Brit. J. Exp. Path.*, 1931, v12, 88.