streptozotocin diabetes. It was postualted that streptozotocin may interfere with NAD formation in the beta cell and that treatment with nicotinamide reverses the streptozotocin effect by reversing its effect on levels of NAD within beta cells.

1. Herr, R. R., Jahnke, H. K., and Argoudelis, A. D., J. Am. Chem. Soc. **89**, 4808 (1967).

2. Evans, J. S., Gerritsen, G. C., Mann, K. M., and Owen, S. P., Cancer Chemotherapy Rept. **48**, 1 (1965).

3. Vavra, J. J., DeBoer, C., Dietz, A., Hanka, L. J., and Sokolski, W. T., "Antibiotics Annual," p. 230. Antibiotica Inc., New York (1959–1960).

4. Lewis, C. and Barbiers, A. R., "Antibiotics Annual" p. 247. Antibiotica Inc., New York (1959–1960).

5. Rakieten, N., Rakieten, M. L., and Nadkarni, V., Cancer Chemotherapy Rept. **29**, 91 (1963).

6. Junod, A., Lambert, A. E., Orci, L., Pictet, R., Gonet, A. E., and Renold, A. E., Proc. Soc. Exptl. Biol. Med. **126**, 201 (1967).

7. Arison, R. N. and Feudale, E. L., Nature **214**, 1254 (1967).

8. Arison, R. N., Ciaccio, E. I., Glitzer, M. S., Cassaro, J. A., and Pruss, M. P., Diabetes **6**, 51 (1967).

9. Schein, P., Cooney, D., and Vernon, L., Cancer Res. **27**, 2324 (1967).

10. Dulin, W. E., Lund, G. H., and Gerritsen, G. C., Diabetes **16**, 512 (1967).

11. Lazarow, A., Liambies, J., and Tausch, A. J., J. Lab. Clin. Med. **38**, 249 (1950).

12. Kaplon, N. O., Goldin, A., Humphreys, S. R., and Ciotti, M. M., Science **120**, 457 (1954).

13. Schiyama, A., Nakamura, S., and Nishizuki, Y., Arzneimittel-Forsch. **17**, 1346 (1967).

14. Schiyama, A., Nakamura, S., and Nishizuki, Y., Arzneimittel-Forsch. **17**, 1525 (1967).

15. Benerjic, S., Science **106**, 128 (1947).

Received Oct. 7, 1968. P.S.E.B.M., 1969, Vol. 130.

The Role of Mycoplasma in Rat Arthritis Induced by 6-Sulfanilamidoindazole* (33708)

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Periarthritis of the hind paws in rats receiving oral 6-sulfanilamidoindazole (6-SAI) was first described by Mielens and Rozitis (1). They reported the isolation of mycoplasma from the inflamed joints using blind passage techniques. However, the isolates were not characterized and serologic studies were not undertaken. Mice which had been similarly treated succumbed to bronchopneumonia. Because old rats and mice may harbor potentially pathogenic mycoplasma (2–6), Mielens and Rozitis suggested that the effects of 6-SAI were due to activation of latent mycoplasmal infections. In a later study by Sigg *et al.* this hypothesis was accepted although no attempt was made to isolate or characterize the suggested etiologic agents (7). In contrast, Ward *et al.* reported preliminary observations indicating that mycoplasma could not be recovered from rats with arthritis induced by 6-SAI (8).

The present study was undertaken to explore in detail the possible role of mycoplasma in 6-SAI arthritis. The principal purpose was to examine for active infection by mycoplasma as the cause of the arthritis.

Materials and Methods. Animals. Male albino rats weighing 400–500 g were obtained from a local supplier or from Holtzman Company (421 Holtzman Road, Madison, Wisconsin). They were housed in individual cages and fed Purina rat chow and tap water *ad libidum.*

Induction of arthritis using 6-sulfanilamidoindazole (6-SAI). Oral administration was carried out using a 15% (w/v) suspen-

* Supported in part by Grants AM-02255 and AM-05016, National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

sion of 6-SAI, in a 1% (w/v) aqueous suspension of gum tragacanth. The animals were fed daily with 250 mg of 6-SAI/kg until arthritis developed. Administration was performed using a gastric tube on animals anesthetized with ether.

Subcutaneous (s.c.) injections of 125 mg of 6-SAI/kg were carried out using 3.0 g of 6-SAI dissolved in 30% (v/v) propylene glycol, 10% (v/v) ethyl alcohol, and 60% normal saline at pH 8.0. Daily injections of 2.0 ml were given until onset of arthritis.

After treatment each animal was examined daily for redness and/or swelling of joints and other evidence of disease. The arthritis was scored as previously described (9).

Induction of arthritis by Mycoplasma arthritidis. *M. arthritidis* was cultured and prepared as previously described (9). Organisms were given by intravenous injection.

Culture media. The following media were used for culture of mycoplasma: (i) KN agar or KN broth consisting of Difco PPLO agar or broth supplemented with 0.25% (w/v) yeast extract (BBL), 0.2 μg/ml of deoxyribonucleic acid, sodium salt (calf thymus, Mann Research Lab., New York), 10% (v/v) horse serum and 1000 units of penicillin/ml; (ii) DPN agar consisting of KN agar with the addition of 0.1 ml/ml diphosphopyridine nucleotide(added from a sterile 2 mg/ml solution: DPN-102, Sigma Chemical Co.); (iii) Sheep blood agar; (iv) KN broth adjusted to pH 6.0; and (v) Thioglycollate medium (BBL, Code No. 01-135.

Isolation procedure. Joints for culturing were skinned and pieces of tissue were removed aseptically and minced with 1–2 ml of sterile Difco PPLO broth containing 10% (v/v) horse serum. Lung tissue was also minced in PPLO broth. The resulting suspensions were plated in duplicate onto KN agar, DPN agar, blood agar, and into thioglycollate broth. Heart blood was plated directly onto these media. Plates were incubated aerobically and anaerobically at 37° and examined at intervals for up to 10 days of incubation.

Blind subcultures were carried out by inoculating 2–3 drops of the tissue suspensions or blood into KN broth, KN broth adjusted to pH 6.0, and DPN broth. After 5 days of aerobic and anaerobic incubation, 0.5-ml amounts of the broths were transferred to fresh media. This was repeated for 8 transfers. Sterile broths were similarly transferred as controls. After the first transfer, all subsequent transfers were plated onto KN agar to check for growth. Isolations from mycoplasma-induced arthritis were carried out by plating joint fluid, lung tissue suspensions, and heart blood onto KN agar only.

Characterization of isolates. Isolates from 6-SAI arthritis were compared with *M. arthritidis*, 14124 (American Type Culture Collection), and *M. arthritidis*, strain PN (isolated by the authors from a spontaneous rat abscess). The biochemical methods used were as described by Cole *et al.* (9). Antisera were prepared in rabbits using the method of Morton and Roberts (10), employing antigens grown in rabbit infusion broth (11). The relationship of strains isolated from 6-SAI arthritis to other mycoplasma strains was investigated by agar gel double diffusion and growth inhibition by antiserum (12).

Antibody studies. Circulating antibody in the rat sera was measured by the complement fixation technique of Kolmer *et al.* (13). Antigens of *M. arthritidis* and *M. pulmonis* were prepared as described previously(14).

Results. Isolation of mycoplasma. Twenty male rats from a local supplier were fed 6-SAI orally until arthritis developed (Expt. 1, Table I). Samples from 3 rats were cultured for mycoplasma at onset and 3 rats at maximum arthritis. Of the latter, both an arthritic joint and a normal joint were cultured. Samples from normal animals were similarly cultured. By the direct plating of suspensions of tissues, mycoplasma appeared in 60% of all lung cultures, including the normal control animals. On the basis of growth characteristics and colonial morphology, these isolates were identified as *Mycoplasma pulmonis*. Growth of these strains was considerably enhanced on the DPN agar. Mycoplasma were not isolated from any of the joint or blood samples by direct plating onto agar. Bacteria were not encountered in

TABLE I. Comparative Effect of Tetracycline on Rat Arthritis Induced by 6-Sulfanilamidoindazole (6-SAI) and *M. arthritidis*.

Experimental group	Route	Incidence of arthritis[a]	Onset of arthritis (days)	Maximum arthritis (days)	Mean score at maximum arthritis	Extremities involved (mean)	Involvement of forelimbs
Expt. 1. Local rats							
6-SAI, 250 mg/kg/day	Oral	20/20	3–5	6–8	15	2	1/20
Effect of re-feeding 250 mg/kg/day	Oral	6/6	2–5	6–8	14	2	2/6
Expt. 2. Holtzman rats							
6-SAI, 125 mg/kg/day	s.c.	5/7[b]	6–7	6–9	6	2	1/7
6-SAI, 125 mg/kg/day; 20 mg/kg of tetracycline i.m. daily	s.c.	7/8[b]	6–9	7–9	5	2	0/8
M. arthritidis, strain PN 2×10^9 cfu	i.v.	10/10	2–3	6–10	26	4	10/10
M. arthritidis, strain PN 1×10^9 cfu with 20 mg /kg of tetracycline i.m. daily	i.v.	0/10	—	—	—	—	—

[a] Number of rats with grossly visible arthritis/number of rats in experimental group.

[b] Rats in each group were sacrificed for culture at onset of arthritis. Because these animals were randomly selected, some did not have arthritis and the proportions shown represent the number with arthritis throughout the experiment.

any of the isolations. In the second blind passage of joint specimens taken at maximum arthritis from all three rats, a few mycoplasmacolonies appeared on the agar transfer from the anaerobic, pH 6.0, KN broths. All three cultures showed colonies with well-defined central areas (Fig. 1A), and one contained, in addition, colonies resembling *M. pulmonis*. The latter could not be maintained in culture. The three isolates were designated 1A, 2A, and 3A, respectively. All of the other cultures were negative for mycoplasma. Mycoplasma did not appear in subsequent blind passages.

During the course of the first experiment, one of the animals developed a small purulent abscess on one of the terminal interphalangeal joints: Mycoplasma were readily isolated from the purulent material by direct plating onto KN agar. This strain was desigeated PJ.

When the arthritis had subsided, a group of rats were retreated with 6-SAI. The onset and severity of arthritis was similar to that obtained during the first feeding of 6-SAI (Expt. 1, Table I). Samples from 6 rats were cultured at maximum arthritis but no growth of *M. arthritidis* was identified.

In a second experiment, Holtzman rats were used. Two groups of 10 rats each were given s.c. injections of 6-SAI. One of the groups was also given daily i.m. injections of 20 mg of tetracycline[1]/kg. The incidence and severity of arthritis was similar in the two groups (Expt. 2, Table I). Joints and heart blood were cultured from control rats and from rats of both groups at onset and maximum arthritis. All cultures were negative for mycoplasma.

For comparison purposes, two groups of 10 Holtzman rats, weighing 400–500 g, were injected with 1×10^9 colony forming units (cfu) of *M. arthritidis*, strain PN. One group was given daily s.c. injections of 20 mg of tetracycline/kg. All animals injected only with *M. arthritidis* developed arthritis, while

[1] Achromycin, Lederle Laboratories.

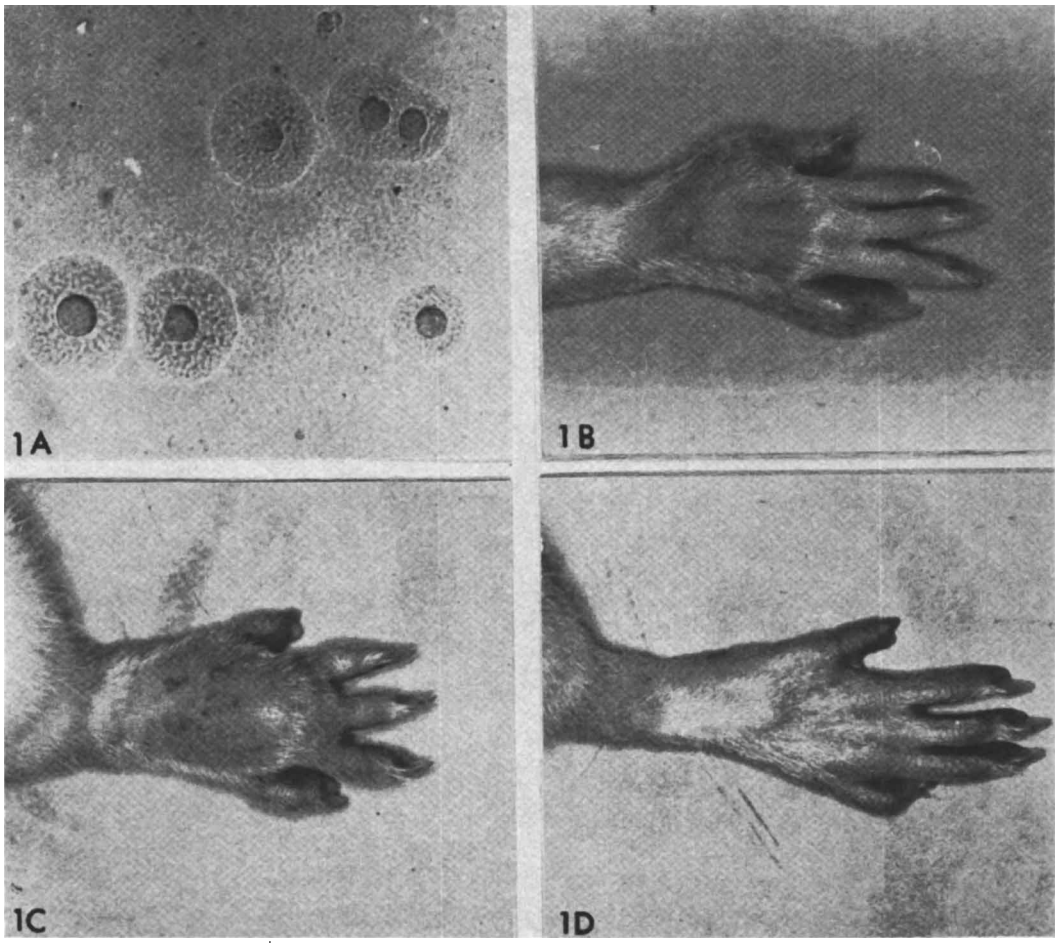

FIG. 1A. Colonies of *M. arthritidis* isolated by blind passaging of a joint sample taken from a rat with 6-SAI-induced arthritis. (B) Arthritis of tibiotarsal, metatarsophalangeal, and interphalangeal joints of a rat hind paw produced by the intravenous injection of a culture of virulent *M. arthritidis*. (C) Diffuse arthritis and periarthritis of a rat hind paw produced by the oral administration of 6-sulfanilamidoindazole. (D) Normal rat hind paw.

those injected with *M. arthritidis* and tetracycline remained healthy (Expt. 2, Table I). Joints and heart blood from three animals in each group at onset and maximum arthritis were cultured directly onto KN agar. In all animals injected only with *M. arthritidis*, mycoplasma appeared in the joint cultures in large numbers, and a few colonies were also present in all cultures of heart blood. The tetracycline treatment completely inhibited the development of the arthritis.

Characterization of isolates. All mycoplasma strains isolated from the joints were found to be identical in their biochemical reactions and growth characteristics to the strains of *M. arthritidis*, with which they were compared (9). All strains were closely related serologically by gel diffusion. The growth of strains 3A and PJ was inhibited when grown in the presence of antiserum prepared against *M. arthritidis*, strains 14124 and 3A.

A suspension of strain 3A was injected intravenously into a group of 6 rats weighing 400–500 g each. An inoculum of 1×10^9 cfu/animal resulted in a 100% incidence of arthritis. The time of onset was 3–5 days, the mean score at maximum arthritis was 13,

TABLE II. Relation between 6-SAI Arthritis and Complement-Fixing Antibody Titers against Mycoplasma.[a]

After initial feeding (days)	No. of animals tested	Mean arthritis scores		Reciprocal of mean complement-fixing titers	
		At maximum arthritis	At time of autopsy	*M. pulmonis* antigen	*M. arthritidis* antigen
2	Control 3	0	0	80	5
	6-SAI 1	0	0	40	5
3	Control 2	0	0	80	<5
4	Control 2	0	0	40	10
	6-SAI 2	11	11	320	5
7	Control 3	0	0	40	10
	6-SAI 2	29	29	80	<5
10	Control 2	0	0	80	<5
	6-SAI 3	23	16	80	5
14	Control 3	0	0	80	<5
	6-SAI	20	8	80	<5
21	6-SAI 4	11	4	80	<5

[a] For comparative purposes, three rats were injected with 1×10^9 cfu of virulent *M. arthritidis*. After 21 days complement-fixing antibody titers of 1:5000–1:20,000 were detected. The mean score at maximum arthritis was 17, and at the time of autopsy (21 days) was 7.

and 5 out of 6 animals had involvement of the forelimbs. Mycoplasma could be readily isolated in large numbers from the joints of these rats by direct plating onto KN agar.

Serologic studies. Three animals from the first experiment were bled at the time of maximum arthritis (6–8 days) and at decline of arthritis (10–14 days). Three control, untreated animals were also bled. Similarly three animals which had received a second feeding of 6-SAI were bled upon decline of the ensuing arthritis. The sera obtained were tested by complement fixation against an antigen of *M. arthritidis* strain 3A. No titers were detected after the first feeding, but 2 out of 3 animals which had been re-fed 6-SAI produced titers of 1:80 and 1:40, respectively, against 3A antigen.

The serologic tests were repreated on the Holtzman rats given 6-SAI only and 6-SAI plus tetracycline (Expt. 2, Table I). Animals were bled prior to administration of 6-SAI, as well as at maximum arthritis and at decline of arthritis. Another group which had recovered from 6-SAI arthritis was re-fed 6-SAI and the animals were bled at maximum arthritis. None of the animals developed antibodies against *M. arthritidis*.

In order to investigate further the possible development of antimycoplasma antibodies in rats given 6-SAI, serum was collected from the animals used in a sequential histopathological study to be reported elsewhere. Groups of Holtzman rats, weighing 430–550 g were sacrificed after 2, 3, 4, 7, 10, 14, and 21 days following the oral administration of 6-SAI. Animals fed gum tragacanth alone served as controls. The sera obtained were tested by complement fixation against antigens of recent isolates of *M. pulmonis* and *M. arthritidis*. The antibody titers obtained, together with the severity of arthritis, are recorded in Table II. Some of the sera were slightly anticomplementary, but all had titers of less than 1:10. All animals including the controls were found to have low liters against *M. pulmonis*. Antibody titers against *M. arthritidis* were negligible. No correlation was found between the presence or absence of arthritis and complement-fixing antibodies against *M. pulmonis* or *M. arthritidis*.

The fact that animals which had recovered

from 6-SAI-induced arthritis were still susceptible to the subsequent administration of 6-SAI, indicated the absence of any immunity. In order to investigate this further, a group of 6 rats weighing 400–500 g recently recovered from the 6-SAI disease, was injected with 1×10^9 cfu/animal of *M. arthritidis*. All developed arthritis which was identical with that produced by the injection of *M. arthritidis* into previously untreated animals.

Gross appearance. Multiple joint involvement usually affecting all four limbs occurred in mycoplasma-induced arthritis. The interphalangeal joints were often involved even when no arthritis was visible in the ankle or metacarpal region (Table I; Fig. 1B). Arthritic joints were usually filled with purulent fluid. In the case of 6-SAI arthritis, involvement of the forelimbs was rare. The interphalangeal joints were rarely involved, but when this occurred, severe arthritis of the ankle or metacarpal joints was always present (Table I; Fig. 1C). Irregular areas of erythema were present in the skin overlaying involved joints.These were absent in mycoplasmal arthritis, in which a generàl inflammation occurred. There was no purulent material in any of the arthritic joints caused by 6-SAI.

Discussion. The data of the present experiments do not support the suggestions of Mielens and Rozitis (1) that the arthritis produced in rats following the administration of 6-SAI was due to the activation of a latent mycoplasma infection. Although mycoplasma were occasionally isolated from the joints of locally supplied animals used in the first experiment, subsequent enquiries and investigations showed that the colony was subject to spontaneous outbreaks of arthritis and that many apparently healthy rats harbored *M. arthritidis*. This would account for the presence of this organism in the 6-SAI-treated animals and for the presence of antimycoplasmal antibodies. It is significant that the Holtzman rats which were used in subsequent experiments were negative in these respects, although arthritis could still be induced with 6-SAI.

Although mycoplasma were regularly isolated from the lungs of most rats examined, the observation is of little significance as older rats commonly harbor *M. pulmonis* (2). The presence of low titers of complement-fixing antibody against *M. pulmonis* in both control and arthritic rats is consistent with this observation. The inability to correlate complement-fixing antibodies against *M. pulmonis* and *M. arthritidis* with the presence or absence of 6-SAI arthritis does not support anetiological role for these organisms in this disease. On the other hand, we have observed that rats injected intravenously with virulent cultures of *M. arthritidis* produce high titers of complement-fixing antibodies against *M. arthritidis* antigen (Cahill, J. F., Cole, B. C., Wiley, B., and Ward, J. R., unpublished observations). The finding that rats which have recovered from 6-SAI-induced arthritis are still susceptible to the further administration of 6-SAI is in contrast to the immunity which develops as a result of mycoplasma-induced arthritis (Cahill, J. F., Cole, B. C. Wiley, B., Ward, J. R., unpublished observations: 15). Furthermore, animals which have recovered from 6-SAI-induced arthritis are as equally susceptible to mycoplasmal arthritis as control untreated animals of the same age.

Tetracycline treatment, which completely inhibited mycoplasma-induced arthritis, had no effect upon that caused by 6-SAI. Furthermore, the distribution of arthritis was markedly different in the two cases. No evidence of suppuration, which is characteristic of the mycoplasmal disease, was present in the 6-SAI-treated animals. The ease of isolation of mycoplasma from arthritis induced by *M. arthritidis* contrasts with the infrequency of isolation from 6-SAI-induced arthritis. Other workers have been unable to isolate mycoplasma from the 6-SAI disease (16). Mielens' isolates were not described or characterized and so it is possible that they could represent some species other than those considered in this paper. That mycoplasma are involved at all is unlikely in view of the observations in regard to tetracycline therapy. *M. arthritidis* and *M. pulmonis* could occasionally occur, as secondary invaders, in

6-SAI-induced arthritis, but they had no significant role in the etiology of this disease.

Summary. Arthritis could be induced in rats by the oral and subcutaneous administration of 6-sulfanilamidoindazole (6-SAI). *M. arthritidis* could be occasionally isolated from involved joints, but the results obtained from tetracycline therapy, histopathology, and serologic studies, in comparison with mycoplasma-induced arthritis, indicated that these organisms were not the etiologic agent of the disease. It was concluded that mycoplasma could appear in 6-SAI arthritis as secondary invaders.

We wish to thank Zigurd E. Mielens (Sterling-Winthrop Research Institute, Rensselaer, New York) for his generosity in making supplies of 6-sulfanilamidoindazole available to us. Thanks are also due to Laura Golightly Rowland and James F. Cahill for technical assistance during portions of this study.

1. Mielens, A. E. and Rozitis, J. Jr., Proc. Soc. Exptl. Biol. Med. **117**, 751 (1964).

2. Klieneberger, E. and Steabben, D. B., J. Hyg. **40**, 223 (1940).

3. Klieneberger-Nobel, E., Ann. N. Y. Acad. Sci. **79**, 615 (1960).

4. Edward, D. G., J. Pathol. Bacteriol. **59**, 209 (1947).

5. Nelson, J. B., J. Exptl. Med. **65**, 843 (1937).

6. Sabin, A. B. and Johnson, B., Proc. Soc. Exptl. Biol. Med. **44**, 509 (1940).

7. Sigg, E. B., Traeme, M. L., and John, M., J. Pharmacol. Exptl. Therap. **157**, 214 (1967).

8. Ward, J. R., Miller, M. L., Cole, B. C., and Cathey, W., Excerpta Med. **E21**, (1967).

9. Cole, B. C., Miller, M. L., and Ward, J. R., Proc. Soc. Exptl. Biol. Med. **124**, 103 (1967).

10. Morton, H. E. and Roberts, R. J., Bacteriol. Proc. **1966**, 139.

11. Taylor-Robinson, D., Somerson, N. L., Turner, H. C., and Chanock, R. M., J. Bacteriol. **85**, 1261 (1963).

12. Clyde, W. A., Jr., J. Immunol. **92**, 958 (1964).

13. Kolmer, J. A., Spaulding, E. H., and Robinson, H. W., "Approved Laboratory Technic," 5th ed. Appleton, New York (1951).

14. Cole, B. C., Golightly, L. and Ward, J. R., J. Bacteriol. **94**, 1451 (1967).

15. Collier, W. A., J. Pathol. Bacteriol. **48**, 579 (1939).

16. Thomas, L., Public Health Serv. Publ. **1523**, (1966).

Received Oct. 9, 1968. P.S.E.B.M., 1969, Vol. 130.