guinea pig, the cat, and probably the rabbit‡ is followed by the chicken and the Rhesus monkey, both of which were extremely resistant, at least to ANTU given by stomach tube.

It is interesting to note that in contrast to the large variations in the toxicity of thiourea to Norway rats from different sources,[5] little difference was found in the response of the same rats to ANTU. Rats from Dr. Astwood's colony in Harvard (designated Strain II) were only twice as resistant as rats from our colony (Strain I), and hardly differed at all from the wild Norways.

The mode of administering the ANTU did not influence the toxicity in rats, but in other species less ANTU was required to kill by intraperitoneal injection than when given by stomach tube. For instance monkeys died following 200 mg/kg of ANTU given intraperitoneally but withstood 17 times that amount by stomach tube, and dogs likewise showed a several-fold difference. In part this may be ascribed to emesis, which occurred fairly often in dogs, cats, and monkeys, but elimination in this manner before absorption could take place was minimized by starving the animals overnight before they received doses by stomach tube, and withholding food until 6 to 8 hours later.

The effects characteristic of ANTU poisoning in Norway rats, namely pulmonary edema and pleural effusion, were not found in some species even after the administration of fatal doses. Examination of sections from the lungs of Alexandrine rats, guinea pigs, rabbits and monkeys has not revealed edema in anything like the amounts found in the lungs of Norway rats, dogs, mice and cats.[10] It would therefore seem likely that the mechanism of action of ANTU is different in these 2 groups of animals.

Both males and females were used in most species tested (the rabbits and chickens were all females) without any striking sex difference in susceptibility becoming evident. Sex differences cannot be ruled out, however, on the basis of these limited data. An indication that an age difference exists in dogs, comparable to that observed in rats, is found in the fact that 3 puppies survived doses which would have been fatal to adult dogs.

Summary. Marked age variation in the susceptibility of wild Norway rats to acute poisoning with α-naphthyl thiourea has been found, with suckling rats about 7 times as resistant as adults. This resistance was found to decrease with increasing body weight, levelling off for adults at an LD_{50} between 6 and 8 mg/kg body weight. The difference found between adult males and females was within the limits of error and is therefore not considered significant.

Adult animals of other species also varied markedly in susceptibility to acute ANTU poisoning. Norway rats, dogs and mice were killed by amounts less than a hundred milligrams per kilogram. Alexandrine rats, guinea pigs, and cats required several hundred milligrams, while chickens and monkeys survived doses of several grams per kilogram body weight. Large amounts of pulmonary edema were found only in Norway rats, dogs, mice and cats.

‡ McClosky and Smith[4] found an LD_{50} for rabbits of about 1000 mg/kg.

[10] Latta, H., unpublished results.

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Bactericidal Action of Streptomycin.

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Streptomycin,[1] which inhibits the growth of a variety of gram positive, gram negative and acid fast microorganisms[1-7] has been found by Waksman and Reilly[8] to be bactericidal for *Escherichia coli, Pseudomonas aeruginosa* and *Proteus vulgaris*. According to Wallace

and co-workers,[9] the bactericidal action of streptomycin on *Staphylococcus aureus* and *Eberthella typhosum* was influenced by the culture medium. Donovick and Rake[10] found that 0.056 units per ml of streptomycin inhibited the growth of 1000 cells per ml of *Klebsiella pneumoniae* in 0.75% tryptone broth and that the minimal inhibiting dose varied with the lot and the concentration of tryptone. The present study of the bactericidal action of streptomycin was made on *K. pneumoniae,** which is used in this laboratory for the bio-assay of streptomycin.[11]

In order to follow the bactericidal action of streptomycin on this organism, 6 ml of a diluted 6-hour culture containing about 5 million cells per ml were mixed with 0.1 ml of streptomycin and incubated at 37 C. Plate counts, made at intervals in 2% tryptone 0.2% glucose agar, are given in Table I. All figures for bacterial counts are averages of 3 plates.

Comparison of the results in yeast beef broth with those in tryptone broth illustrates the effect of culture medium on the activity of streptomycin. While 2 units per ml in yeast beef broth retarded growth slightly, 1 unit per ml in tryptone broth produced a 500,000-fold reduction in count in one hour. Although different preparations of streptomycin were used, this could not account for these results, because repeated experiments in the same medium have failed to show any difference between the activity of pure and impure streptomycin.

The bactericidal effect of 1.0 unit of streptomycin per ml in tryptone broth was evident after 20 minutes incubation. On the other hand, 0.25 unit per ml caused no reduction in count during the first hour of incubation, but, following this induction period, the count dropped 60,000-fold in 3 hours, and after 24 hours incubation, rose to about one-seventh of that in the control tube. This rise in count, which also occurred in tryptone broth containing 0.5 unit per ml of streptomycin, was contrary to expectations because 1000 cells per ml in tryptone broth are inhibited by 0.056 unit per ml. Two possible explanations for the rise in count are: (1) that during the 3-hour incubation period in the presence of large numbers of cells, the streptomycin was used up, so that the concentration fell below the minimal inhibitory level; or (2) that the cells surviving at the end of 3 hours incubation with streptomycin were more resistant to its action. Experiments were set up to test the latter hypothesis. Plate counts were made in 0.75% tryptone agar with and without streptomycin. Results of these experiments are given in Fig. 1.

Although there were a few cells in the control tube resistant to 1.0 units and 3.0 units of streptomycin, the proportion of resistant to total cells in this tube remained small throughout the experiment. However, in the tube containing 0.25 unit of streptomycin per ml, the number of resistant cells in comparison to total cells, which was small after 3 hours incubation, became almost equal after 6 hours incubation and remained so through 12 and 16 hours incubation. (Exp. 2, Fig. 1). In a third experiment, when the concentration of streptomycin in the agar was increased to 4.9 and 49 units per ml, almost all the organisms surviving the bactericidal

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[2] Schatz, A., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 244.

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[4] Robinson, H. J., Smith, D. G., and Graessle, O. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 226.

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[6] Waksman, S. A., Reilly, H. C., and Schatz, A., *Proc. Nat. Acad. Sci. U. S.*, 1945, **31**, 157.

[7] Waksman, S. A., and Schatz, A., *J. Am. Pharm. Assn. Sci. Ed.*, 1945, **34**, 273.

[8] Waksman, S. A., and Reilly, H. C., *J. Inf. Dis.*, 1944, **75**, 150.

[9] Wallace, G. I., Rhymer, I., Gibson, O., and Shattuck, M., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 127.

[10] Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 224.

* American Type Culture Collection No. 9997.

[11] Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, **50**, 623.

TABLE I.
Bactericidal Action of Streptomycin on Multiplying Cultures of *Klebsiella pneumoniae*. (No. of Organisms per ml × 1000).

Incubation time	Yeast beef broth Units per ml impure streptomycin 504			
	4	3	2	0
0				6430
2 hr	17.6	>3000	46300	84600
3 "	0.8	2120	127000	212000
4 "	0.1	154	443000	836000
23 "	820000.	865000	481000	1,150000
	0.75% tryptone broth Units per ml pure streptomycin M208E			
	1.0	0.5	0.25	0
0				6760
20 min	4000.	7000.	7360.	6800
40 "	7.16	3760.	8030.	9700
1 hr	0.01	50.3	>3000.	16200
2 "	0.00	0.04	>3.0	75300
3 "	0.00	0.01	0.08	195000
24 "	0.00	122000.	109000.	753000
0				7250
1 hr		12.0	4580.	21900
2 "		0.03	1.18	67400
3 "		0.02	0.00	144000
4 "		0.03	0.07	145000
28 "		136000.	61400.	258000

effect of 0.25 unit per ml in broth after 12 hours incubation grew in plates containing 4.9 units per ml, but none grew on plates containing 49 units per ml. Apparently, then, this resistance which was acquired by this organism, or was a result of selection, is not of a high order of magnitude.

To determine whether or not this resistance was a temporary characteristic, transplants were made in the third experiment from the tube containing 0.25 unit per ml in broth after 0 hours and 16 hours incubation, to tubes containing 0.0 unit, 0.07 unit, 0.35 and 1.75 units of streptomycin per ml of tryptone broth. Serial subcultures were made after 24 hours incubation from the tube containing no streptomycin to tubes with and without streptomycin. The results of these experiments are given in Fig. 2 Three things are apparent: (1) After 16 hours incubation in broth containing 0.25 unit per ml, the organisms grew in broth containing 5 times as much streptomycin as the minimal inhibiting dose for the same culture after 0 hours. This was to be expected, since at that time the plate count in agar containing 4.9 units of streptomycin per ml was almost equal to that in agar without streptomycin (Fig. 1). (2) The amount of streptomycin in broth to which these organisms were resistant after 16 hours incubation was much less than that in agar (0.35 unit per ml compared to 4.9 units per ml, Fig. 1 and Fig. 2); (3) after 3 subcultures in the absence of streptomycin, the 2 cultures retained their respective characteristics, an indication that a persistent change in the resistance of the organism had occurred.

Some evidence was also obtained for the appearance of resistance in *Klebsiella pneumoniae* by repeated subculturing in tryptone broth containing streptomycin. After 2 serial subcultures in 0.35 unit of streptomycin per ml, the resistance of the organisms taken originally from broth containing 0.25 unit per ml after 16 hours incubation rose from 0.35 unit per ml to 1.75 units per ml. (Fig. 2). However, this 5-fold increase in resistance is slight in comparison with the increase in resistance of gonococci and meningococci from about 40 units per ml to 75,000 units per ml which Miller and Bohn-

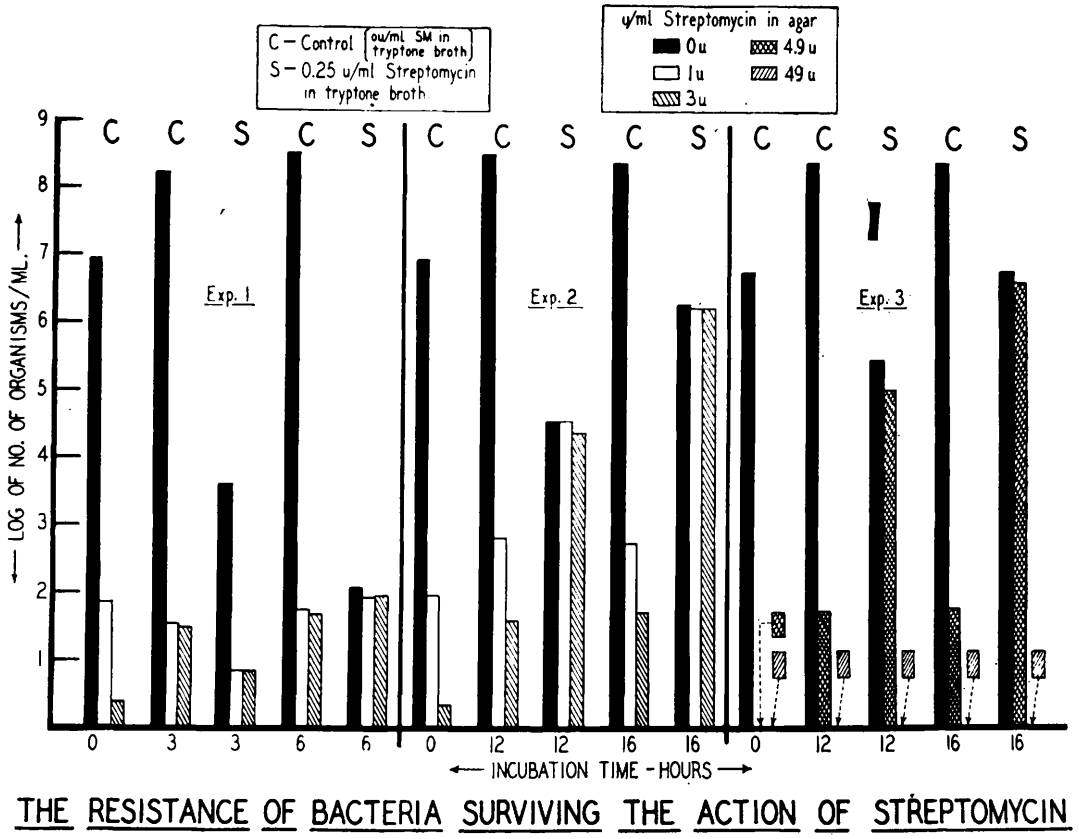

Fig. 1.

TABLE II.
Bactericidal Action of Streptomycin on Washed Cultures of *Klebsiella pneumoniae*.
(No. of Organisms per ml × 1000).

Incubation time	Units per ml pure streptomycin M208E			
	30	15	7.5	0
0				5760
20 min	1100.	5300.	5530.	6100
40 ''	3.55	616.	5000.	5730
1 hr	.02	10.4	1983.	5430
2 ''	<.01	.023	12.9	4900
3 ''	<.01	.013	.066	7300
	15	7.5	3.75	0
0				5700
20 min	5600.	6100.	6100	5230
40 ''	2590.	4230.	5830	5430
1 hr	220.	3360.	5600	5560
2 ''	.00	108.	2050	7960
3 ''	.00	.00	172	7800
		3.78	1.89	0
0				4530
2 hr		5260	5860	5700
4 ''		3240	6800	7600
6 ''		443	6130	8130

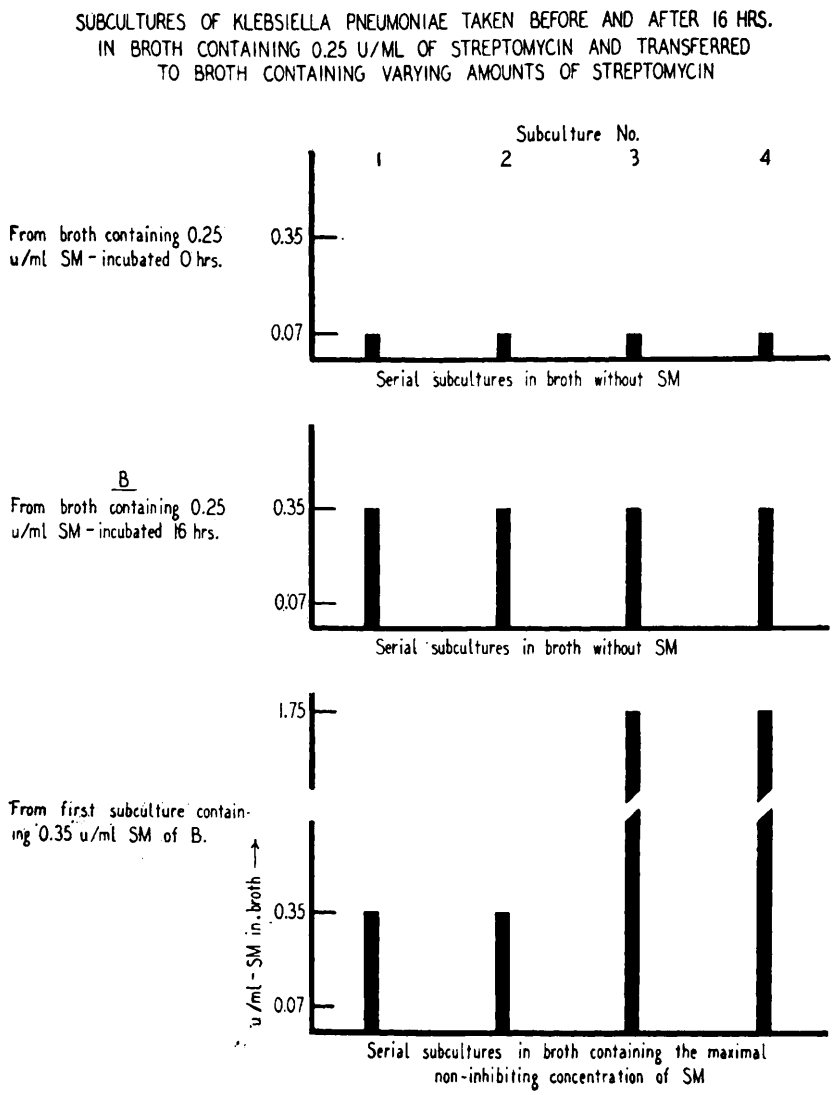

Fig. 2.

hoff[12] were able to obtain by serial transfer on agar containing streptomycin.

The bactericidal action of streptomycin on non-multiplying cultures of *K. pneumoniae* was next investigated. A 6-hour culture was washed 3 times by centrifuging with Ringer's solution, resuspended in Ringer's solution to give approximately 10 million cells per ml and mixed with an equal volume of streptomycin in M/15 phosphate buffer pH 6.8. During incubation at 37°C plate counts were made in 2% tryptone 0.2% glucose agar. Results of average counts of 3 plates are given in Table II.

Thirty units per ml reduced the number of viable cells greater than 5-fold after 20 minutes incubation, 3.75 units per ml caused a slight drop in count after 3 to 6 hours incubation, but 1.89 units per ml had no effect. These results indicate that the mode of action of streptomycin differs from that of penicillin, which had no action on

[12] Miller, C. P., and Bohnhoff, M., *J. Am. Med. Assn.*, 1945, **130**, 485.

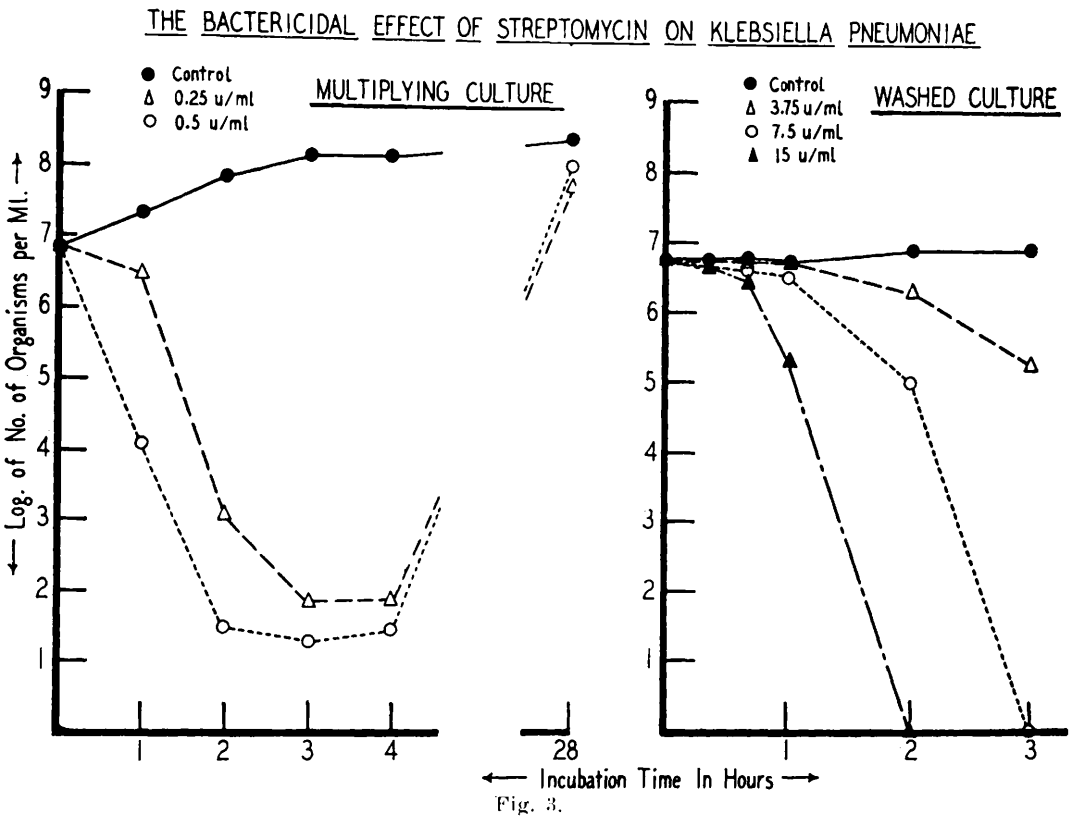

Fig. 3.

a washed culture of *Staphylococcus aureus*, Heatley strain, even at 1000 units per ml.[13]

Because the amount of streptomycin required to produce a drastic drop in the number of viable cells in a washed, non-multiplying culture is much greater than that required for a multiplying culture (Fig. 3), it seems probable that the mode of action of streptomycin is different for washed, non-multiplying bacteria.

It will be recalled that with multiplying cultures almost all the organisms surviving 6 hours incubation with 0.25 unit of streptomycin per ml were more resistant to streptomycin. In experiments with washed cultures, incubated with 7.5 units per ml and plated in agar containing 1.0 and 3.0 units of streptomycin per ml, no evidence was obtained to indicate that the organisms surviving 3 hours or 5 hours incubation were more resistant than the control. This also favors the theory that the mode of action of streptomycin on washed organisms is different from that on multiplying cultures.

In view of the bactericidal action of streptomycin on washed cultures of *K. pneumoniae*, it was of interest to determine whether or not streptomycin had any action on washed spores of a susceptible species. It was found that 43,100 units per ml in buffered Ringer's solution had no effect during 6 hours or 24 hours incubation with washed spores of an unidentified gram positive bacillus (*Bacillus sp.* No. 290). Multiplying cultures of this organism were very sensitive to streptomycin, being inhibited by as little as 0.013 unit per ml in tryptone broth containing 1500 cells per ml.

Summary. Streptomycin was bactericidal for both multiplying and non-multiplying cultures of *K. pneumoniae*, but not for washed spores of *Bacillus sp.* No. 290. Organisms surviving the bactericidal effect of 0.25 unit of

[13] Todd, E. W., *Lancet*, 1945, **1**, 74.

streptomycin per ml after 6 hours incubation were more resistant to its action. This resistance persisted through 4 subcultures in the absence of streptomycin and could be increased 5-fold by serial transfers in broth containing streptomycin. Washed organisms surviving after 5 hours incubation with 7.5 units of streptomycin per ml were not more resistant.

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A Procedure for Testing Sterility of Concentrated Streptomycin Solutions.

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Since the use of streptomycin as a therapeutic agent is rapidly increasing it is important to be able to test the sterility of highly concentrated solutions of this antibiotic. In such sterility tests it is, of course, a prerequisite that the sample of streptomycin taken be of sufficient volume to be representative of the lot of streptomycin involved. As the bacteriostatic levels of streptomycin, at least in the case of many organisms, differ greatly from bactericidal concentrations[1] the addition of such an adequate sample of streptomycin to the sterility test broth may result in inhibition of growth of any viable cells which may be present. Hence there is need for a method of inactivating streptomycin without destroying any living organisms present.

In order to test the sterility of streptomycin solutions in this laboratory, a procedure has been devised which incorporates the inactivation of streptomycin with semicarbazide[2,4] and the use, as the culture medium, of thioglycolate broth which interferes with streptomycin activity.[3]

Of the carbonyl reagents which inactivate streptomycin, semicarbazide was chosen because it is one of the least bacteriostatic members of this group (Table I) and is readily soluble in water. Thiosemicarbazide is also relatively low in bacteriostatic activity but is much less soluble.

TABLE I.
Comparative Sensitivity of Various Organisms to Streptomycin and to Certain Carbonyl Reagents.

Organism	Growth inhibiting concentration in 1% tryptone broth					
	Streptomycin mg/ml	HA-HCl mg/ml	HZ-H_2O mg/ml	SC-HCl mg/ml	TSC mg/ml	Ratio SC-HCl/streptomycin
K. pneumoniae (A.T.C.C. No. 9997)	0.000055*	0.0088	0.0068	0.073	0.116	1325
E. coli No. 33	0.00012*	0.016	0.010	0.064	0.088	533
Staph. aureus (Heatley)	0.000049*	0.018	0.017	>0.54	0.017	>11000
B. subtilis No. 558	0.000056*	0.012	0.012	0.48	0.23	8570
Bacillus sp. No. 290	0.000013*	0.010	0.0073	0.089	0.15	6840

* Based on pure streptomycin base as having 1000 units per mg.
HA-HCl = hydroxylamine hydrochloride.
HZ-H_2O = hydrazine hydrate.
SC-HCl = semicarbazide hydrochloride.
TSC = thiosemicarbazide.

[1] Hamre, D., Rake, G., and Donovick, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 25.

[2] Brink, N. G., Kuehl, F. A., and Folkers, K., *Science*, 1945, **102**, 506.

[3] Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 224.

[4] Donovick, R., Rake, G., and Fried, J., *J. Biol. Chem.*, in press.