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A Microbiological Assay for Isonicotinyl Hydrazine. (19709)

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Two essentially different chemical methods have been described for the determination of isonicotinyl hydrazine in biological fluids. The method of Kelly and Poet(1) is dependent on the reaction of the hydrazine portion of the drug with paradimethylaminobenzaldehyde. In the procedure of Rubin *et al.* (2), isonicotinyl hydrazine is decomposed quantitatively into isonicotinic acid and the latter compound is determined by a method employing cyanogen bromide. Since neither chemical assay is specific for the unaltered drug, it was thought that a microbiological method, having a somewhat higher degree of specificity, would be desirable. In the presently described assay, isonicotinic acid is completely inactive and hydrazine has only 1/50 the activity of isonicotinyl hydrazine.

Experimental. Preparation of the assay plates. The test organism employed is a strain of *Mycobacterium butyricum,* obtained from the ATCC in 1943 as their culture No. 362. Stock cultures of the organism are maintained on slants of the following composition:

	g
Glucose	5
Bacto peptone	5
" yeast extract	5
" beef "	5
" agar	17.5
Distilled water to make 1 liter	

The pH is adjusted to 6.8 before sterilization. Abundant growth is obtained after 24 hours incubation at 37°C. Stock slants may be refrigerated for several months without impairing their usefulness. Inoculum for seeding the assay agar is prepared in a medium of the following composition:

	g
Bacto casamino acids	6
Sodium acetate	1
Trigamine‖	.45
dl-tryptophane	.20
Magnesium sulfate hepta hydrate	.10
Dipotassium phosphate	.10
Calcium nitrate	.08
Ferric chloride, hexahydrate	1.25 mg
Copper chloride dihydrate	1 mg
Manganese chloride hexahydrate	.05 mg
Zinc chloride	.05 mg
Glucose (added aseptically after sterilization)	10 g
Distilled water to make 900 ml	

The pH of the medium is adjusted to 6.8 before sterilization and 18 ml amounts are portioned into 125 ml Erlenmeyer flasks. Sterilization is effected at 120°C for 15 minutes. When cool, 2 ml of sterile 10% glucose solution are then added to each flask. For inoculation of the liquid medium a liberal quantity (about one-fourth of a 3 mm loop full) of growth is removed from a stock slant and suspended evenly in the medium. The inoculum is ready for use after 18-24 hours of incubation at 37°C.

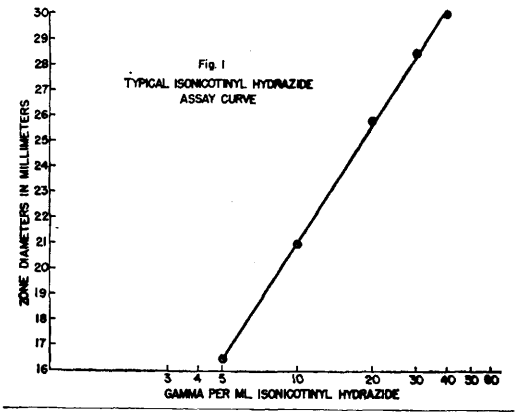
The agar used for pouring the assay plates has the same composition as the liquid inoculum medium except for the inclusion of 1.5% agar. Five ml of this medium are poured into Corning pressed flat-bottom culture plates No. 3162 and allowed to harden. Four ml of the same medium, seeded with 3% v/v of 18-24 hour inoculum, are then carefully and evenly layered over the cooled 5 ml of un-

* Contribution 318.

‖ Obtained from Glyco Products Co., Brooklyn, N. Y.

seeded base agar. For these operations the plates are maintained in a perfectly level position and the agar is held at 48-52° for pouring. When the seed layer has hardened, the assay cups,† previously sterilized, are placed on the agar surface.‡ Gentle heating of the cups to permit sealing to the agar will be found advantageous in the handling of the plates(3). All cups must be heated evenly and uniformly, however, otherwise irregularity of size or distortion of inhibition zones may result. We have found the technic of Schmidt and Moyer(4) convenient and employ 5 cups per plate, using 2 cups for a standard solution containing 20 γ per ml and 3 cups for the sample. The use of 5 replicate plates permits an assay reproducibility of about ± 15% and higher precision can be obtained by the use of a larger number of replicate plates.

Preparation of the sample. To 10 ml of urine in a suitable sized test tube is added 1 ml of concentrated hydrochloric acid. After thorough mixing, the tube is covered to reduce evaporation and held in a boiling water bath for 20 minutes. The sample is then cooled, adjusted to pH 6.5-7.5, and is then diluted to contain from 10 to 30 γ per ml of drug. Equal portions of the sample are then placed in the cups for assay and the plates are incubated at 37°C. The inhibition zone diameters may be read after 18-24 hours.



† Steatite cylinders, 1 mm wall thickness, 8 mm o.d. x 10 mm long, manufactured by Steatite Corp. of America, Keasby, N. J.

‡ When the quantity of the sample is limited, paper discs (Schleicher & Schull No. 740-E) may be used in place of the porcelain cups. The assay curve must then also be set up with paper discs to maintain proportionality.

TABLE I. Analytical Recovery of Known Additions of Isonicotinyl Hydrazine.

Sample	% recovery from urine after addition of 30 γ	100 γ
1	—	93
2	124	100
3	97	97
4	92	100
5	87	91
6	87	102
7	77	97
8	100	98
9	72	88

TABLE II. Comparison of Isonicotinyl Hydrazine Values Obtained by Chemical and Microbiological Assay of Urines.

Sample	Oral dose, mg	Mg isonicotinyl hydrazine excreted as determined by: Microbio assay	Chemical(5) assay
1	125	42	61
2	125	68	65
3	125	51	65
4	190	48	58
5	190	63	79
6	125	50	59
7	190	72	58
8	190	22	48
9	125	29	63

Calculation of results. The assay values are calculated from a standard curve set up according to the method of Schmidt and Moyer (4) with each day's samples. A typical dose-response curve for the concentration range of 5-40 γ per ml is shown in Fig. 1.

Discussion. The method described herein has been found useful in assaying isonicotinyl hydrazine in urines from human and animal subjects submitting to drug excretion studies. As seen from Table I, most samples studied permit good analytical recoveries of known increments of isonicotinyl hydrazine. Moreover, as shown in Table II, fair agreement is obtained with the cyanogen bromide method (2) in the assay of urines from normal subjects.§ These data were obtained on typical

§ We are indebted to Mr. E. DeRitter and to Mr. L. Drekter for access to their data, obtained by the cyanogen bromide assay.

TABLE III. Effect of Hot Acid Treatment on Microbiological Assay of Urines.

Sample	γ/ml isonicotinyl hydrazine found	
	Before treatment	After treatment
1	48	45
2	13	53
3	31	44
4	5	40
5	10	21
6	6	28
7	11	61
8	19	17
9	25	19

24-hour pooled urine specimens, contributed by 9 normal human subjects who had taken orally 125 mg or 195 mg of isonicotinyl hydrazine. The acid-heat treatment is required to release the maximum anti-microbial activity from most urine specimens studied. Occasionally samples are encountered which do not require the acid-heat treatment, such as sample 1, and others are encountered (samples 8, 9) which, even after treatment, do not release all the anti-microbial activity indicated to be present by chemical assay. These data may be seen from Table III. The microbiological activity of aqueous solutions of isonicotinyl hydrazine is not altered by the acid-heat treatment.

Hydrazine has less than 2% the activity of isonicotinyl hydrazine in this assay and isonicotinic acid and isonicotinamide are completely inactive. Combinations of hydrazine and isonicotinic acid have no more activity than that of hydrazine alone. Drug-free urines, when treated as in the isonicotinyl hydrazine assay, have also been found to be completely inactive. Interference will be observed, however, if the urines contain streptomycin, dihydrostreptomycin, aureomycin, terramycin or chloromycetin. Paraamino salicylic acid causes no interference at a concentration of 1000 γ per ml and penicillin is without effect.

Further studies to increase the usefulness of the method are in progress.

Summary. A simple microbiological diffusion-plate assay for isonicotinyl hydrazine has been devised employing *Mycobacterium butyricum* ATCC No. 362 as the test organism. The method is suitable for assaying urines and other aqueous solutions containing 5 γ per ml or more of the drug. The solutions need not be sterile. The assay may be read in 18-24 hours and results are reproducible to ± 15% or closer. Results obtained by this method are in fair agreement with those obtained by the cyanogen bromide chemical assay.

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Stimulation of Plant Growth by Antibiotics. (19710)

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Preliminary experiments indicate that under appropriate conditions antibiotics can stimulate the growth of plant tissues, plant organs and whole plants. Although the extent of this phenomenon is at present unknown, tests are under way to determine which plants are affected and which antibiotics are effective. The present communication presents evidence for plant growth stimulation by antibiotics from 3 types of experiments.

Materials and methods. Tissue culture. The material used in these *in vitro* experiments was the virus tumor tissue from the root of the sorrel plant (*Rumex acetosa*).