pearance of many fine granules, fraying and disruption of the cellular membrane, granule scattering, and, finally, cellular destruction and disappearance. The encouraging results obtained in the treated animal warrant further investigation of cortisone in the treatment of dogs with malignant mast cell tumors.

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Urinary Excretion of Hydrazine Derivatives of Isonicotinic Acid in Normal Humans.* (19476)

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Recent reports have shown that isonicotinyl hydrazide (Rimifon) and 1-isonicotinyl-2-isopropyl hydrazine (Marsilid) are effective antitubercular agents in mice(1,2), guinea pigs and rabbits(3), monkeys(4), and humans(5-8). We have described an assay method and determined levels of Rimifon and Marsilid in blood plasma after oral dosage to humans, dogs and mice(9). In this method, the hydrazides are converted by permanganate treatment to isonicotinic acid, which is determined colorimetrically by the reaction with 10% cyanogen bromide and ammonia used by Mueller and Fox(10) for nicotinic acid assay. Preformed isonicotinic acid is determined by assay *before* permanganate oxidation. In the experiments described below, the cyanogen bromide-ammonia reaction has been applied to untreated and permanganate-treated urines to study the products and rate of excretion by normal humans of oral doses of Rimifon and Marsilid.

Experimental. Nine normal subjects (laboratory personnel), 2 females and 7 males ranging in age from 26 to 43 and in weight from 56 to 82 kg, participated in the urine excretion tests. To determine the color produced by normal urines in the cyanogen bromide reaction, pre-dosage control urines were collected on each of 2 successive days. Collections were started at 9 A.M. and made at intervals ending after 1, 2, 3, 8 and 24 hours. On the test days, a single oral dose of Rimifon or Marsilid was given at 9 A.M. and urine collections made at the same intervals for 24 hours. A pooled sample was collected by each subject for the second 24 hours after dose. Diets were not controlled, but the subjects were instructed to follow as nearly as possible the same diet on each of the collection days. In the first test, the subjects were divided into 2 groups and given 250 mg of Marsilid or 125 mg of Rimifon. These doses were reversed in all subjects in the second test; in the third test, all subjects received 192 mg of Rimifon, which is equivalent on a molar basis to 250 mg of Marsilid. At least one week was allowed between successive doses. Both control and test urines were assayed directly and also after permanganate treatment, using the reaction with 10% cyanogen bromide in ammonia buffer as previously described(9). For direct assay, 1 ml of urine plus 5 ml of buffered ammonia reagent were

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TABLE I. Relative Magnitude of Blanks in Control Urines and Isonicotinic Acid Levels in Test Urines. (All values are expressed as mg of "isonicotinic acid" and represent averages for 9 subjects).

	Direct colorimetry					Colorimetry after $KMnO_4$ treatment				
	Blank in control urines			Free isonicotinic acid in test urines		Blank in control urines			Total isonicotinic acid in test urines	
Hr	1st day	2nd day	Dev. from mean	After Rimifon*	After Marsilid*	1st day	2nd day	Dev. from mean	After Rimifon*	After Marsilid*
1	1.92	1.29	.32	3.3	2.9	1.66	1.23	.22	8.6	3.8
2	1.40	1.61	.11	5.5	5.9	1.15	1.65	.25	13.3	7.7
3	1.86	1.39	.24	4.5	6	1.68	1.55	.07	10	7.6
3–8	6.8	6.3	.25	15.3	19.4	6.9	5.7	.6	36.7	26.2
8–24	13.4	17.3	2	18.8	35.2	12.9	13.5	.3	34.3	47.7
Total	25.4	27.9	1.3	47.4	69.4	24.3	23.6	.4	102.9	93
24–48	"	"	"	5.5	12.6	"	"	"	6.5	16.9

* Oral dose of Rimifon = 192 mg and of Marsilid = 250 mg; in each case the dose is equivalent to 172 mg of isonicotinic acid.

diluted to 25 ml of water. After mixing and centrifuging, a 2 ml aliquot was taken for colorimetry.

For the permanganate treatment (to split Rimifon or Marsilid to isonicotinic acid), 1 ml of urine in a 50 ml beaker was diluted with 10 ml of water. Four percent potassium permanganate was added dropwise until a definite pink color persisted and the solution boiled down slowly (in about 30 minutes) to a volume of about 5 ml. The addition of permanganate was repeated during the heating, if necessary to maintain a pink color. After heating, 1% ascorbic acid was added to decolorize and the solution transferred quantitatively to a 25 ml graduate. Five ml of buffered ammonia reagent were added before dilution to 25 ml with water. A part of this solution was centrifuged for 5 minutes at 2000 r.p.m. and a 2 ml aliquot of the clear supernatant taken for colorimetry. The *galvanometer reading* for each urine sample was converted into mg of "isonicotinic acid" on the basis of isonicotinic acid standard readings, which were taken at frequent intervals. Subtraction of the average control value for a given sample from the corresponding test value gives the level of "isonicotinic acid" in the test urine. Pending isolation and positive identification of the substances measured by the above colorimetric tests, which are being investigated by ion exchange and chromatographic methods, the difference between test and control values is referred to as "free isonicotinic acid" for the direct assay and as "total isonicotinic acid" (*i.e.* free isonicotinic acid plus the amount formed on splitting Rimifon and Marsilid) for the assay after permanganate treatment. The increase in color upon treatment is taken therefore as a measure of Rimifon or Marsilid excretion, although it must be pointed out that the product measured may be a conjugated form of the drug or a related substance which behaves similarly in the cyanogen bromide reaction, *i.e.* one which gives practically no color until treated with permanganate. In calculating free isonicotinic acid levels, a correction has been made in each case for the slight color obtained from the Rimifon or Marsilid *per se*(9).

The validity of the estimation of isonicotinic acid in test urines is dependent upon the day-to-day reproducibility of the blank color in normal urines. Table I shows the average blank values for the two control days and gives a comparison of deviations from the mean blank with increases found after ingestion of Rimifon or Marsilid. Although the blank values are high, the average day-to-day variations are small in relation to the increases found in the test urines. Hence, the average test excretions shown are subject to a relatively small error due to blank variations in direct colorimetry and are practically unaffected by these variations after permanganate treatment.

Results. The excretion patterns are shown graphically in Fig. 1, 2 and 3 for doses of

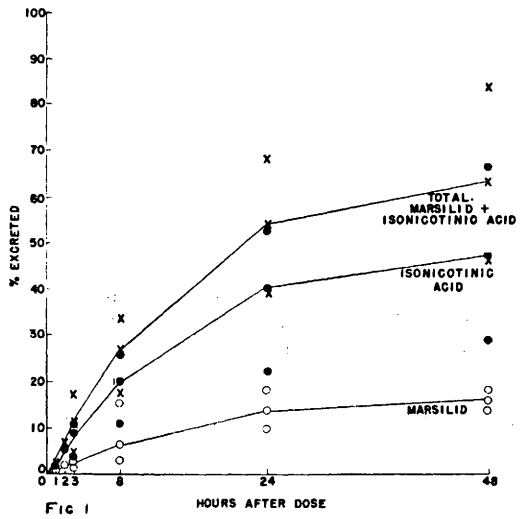

FIG 1

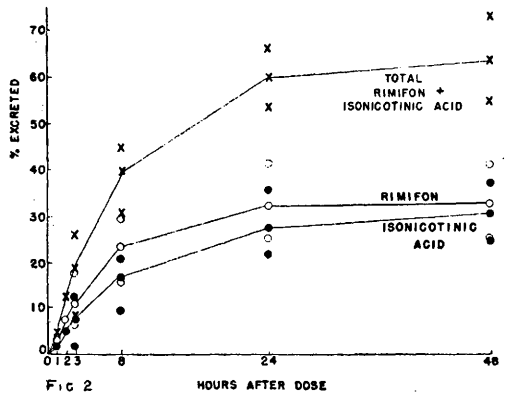

FIG 2

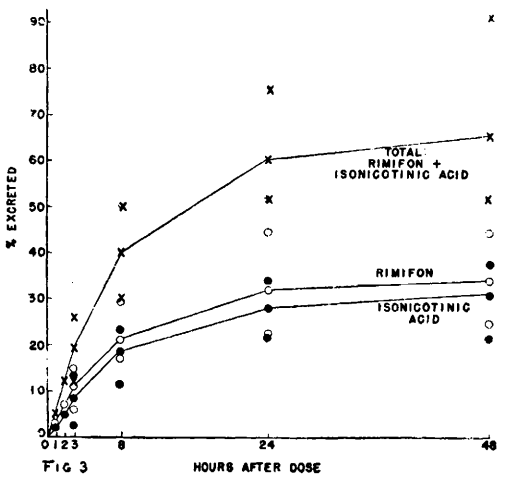

FIG 3

FIG. 1. Average and range of human urinary excretions by 9 normal subjects after an oral dose of 250 mg of Marsilid.

FIG. 2. Average and range of human urinary excretions by 9 normal subjects after oral dosage of 192 mg of Rimifon.

FIG. 3. Average and range of human urinary excretions by 9 normal subjects after an oral dose of 125 mg of Rimifon.

250 mg of Marsilid and 192 and 125 mg of Rimifon, respectively. The lines represent average excretions and the individual points on either side the corresponding minimum and maximum levels for the 9 subjects. The average percentage excretion of Rimifon is greater than of Marsilid, the ratio being higher than 4:1 in the first 3 hours after dose and ranging down to about 2:1 for the total 48-hour excretion. Since the percentage excretion of free isonicotinic acid is practically the same in the first 8 hours after both Rimifon and Marsilid, the total absolute excretion after Rimifon is greater in this period. On the basis of the greater retention of Marsilid, higher blood levels of the latter might be expected. Preliminary assays of blood plasma in this laboratory confirm the fact that levels of Marsilid are higher than of Rimifon after equimolar dosage in humans(11). Similarly, in dog plasma studies(9), in which equal oral doses on a weight basis (*i.e.* a smaller dose of Marsilid on a molar basis) were given, Marsilid showed higher maximum values as well as higher levels after 4 and 8 hours. On a 24- or 48-hour basis, a higher percentage of Marsilid is split and excreted in the urine in the form of isonicotinic acid; this difference between Rimifon and Marsilid may be due to the longer retention of the latter in the body, which allows more time for the splitting to occur. After 48 hours, the total percentage excretion is practically the same after both compounds. The excretion patterns at the two dose levels of Rimifon (Fig. 2 and 3) are almost identical.

Discussion. Although the reaction with 10% cyanogen bromide in ammonia buffer is not specific for isonicotinic acid, it has the advantage of providing a means of differentiating the latter from either Rimifon or Marsilid, which give only a very small percentage of the color obtained with an equivalent amount of isonicotinic acid. An adequate correction for non-specific color in normal urines has been obtained by assaying parallel control samples before dosage. The metabolite responsible for the increased color on direct

assay after dosage has been referred to as isonicotinic acid, but the latter has not yet been isolated and identified. The substance measured is obviously not the unchanged hydrazide since Rimifon or Marsilid *per se* could account for only a small fraction of this color. It is also not niacin or niacinamide since the reaction with 3% cyanogen bromide and Metol, which measures the vitamin or its amide but not isonicotinic acid(9), did not yield significantly greater color with the test urines than with the controls. It is also not an N^1-methyl derivative of isonicotinic acid or its hydrazide since the methiodide of isonicotinic acid hydrazide (Ro 2-5027) gave no color either before or after permanganate treatment. Another possible metabolite is isonicotinamide, but the latter yields only about one-half the color given by isonicotinic acid in the 10% cyanogen bromide-ammonia reaction. Since the 48-hour excretion calculated as isonicotinic acid ranged as high as 67% of the dose, the color can obviously not be due entirely to isonicotinamide. On the basis of the above evidence, it appears likely that the direct assay measures either isonicotinic acid or a mixture of the latter and isonicotinamide. The possibility exists, however, that there may be present other closely related derivatives which are capable of reacting similarly with 10% cyanogen bromide and ammonia. The identity of the component in the test urines responsible for the increased color after permanganate treatment has not been established, but this behavior is typical of both Rimifon and Marsilid. Isonicotinamide shows no such increase in color. Pending further investigation, the colorimetric response has been calculated as Rimifon or Marsilid. It is possible that the substances measured may be conjugated or otherwise modified forms of the hydrazides. If the compound determined after permanganate treatment is isonicotinic acid, then the total percentage excretions shown in Fig. 1, 2 and 3 will remain unchanged regardless of the nature of the excretion products.

It is noteworthy that Marsilid is excreted at a much lower rate than Rimifon in the first 8 hours after dose. The effect of this higher retention of Marsilid on the relative blood levels of both substances is being investigated and preliminary evidence indicates that plasma levels of Marsilid in tubercular patients reach higher peaks and are of longer duration than the levels of Rimifon after equimolar oral doses(11). The significance of this difference in relation to the anti-tubercular activities of the two compounds remains to be evaluated.

Summary. Urinary excretions after oral dosage of Rimifon and Marsilid to 9 normal humans have been studied colorimetrically by means of the reaction with 10% cyanogen bromide in ammonia buffer. The influence of non-specific chromogens has been eliminated to a large degree by parallel assays of predosage control urines. The concentration of metabolite in which the hydrazide linkage has been split was determined by direct assay and the results expressed as "free isonicotinic acid" pending positive identification. The increase in colorimetric readings upon permanganate treatment has been ascribed to unchanged Rimifon or Marsilid, although, again, the exact nature of these excretion products has not been ascertained. Total excretions in 48 hours average about 65% of the dose of both Rimifon and Marsilid, but in the same period the excretion of unhydrolyzed Marsilid is only about half that of Rimifon. In the first few hours after dose, the relative rate of excretion of Marsilid is still lower, averaging only about one-fourth that of Rimifon. Approximately half of the Rimifon is split prior to excretion and almost identical excretion patterns are found at the 192 and 125 mg dose levels. In case of Marsilid, the excretion of free isonicotinic acid parallels closely its excretion after Rimifon for 8 hours, but the 24- and 48-hour totals of free isonicotinic acid are about 50% higher after Marsilid. This increased excretion of the split product results apparently from the longer retention of Marsilid in the body.

Addendum. While the present manuscript was in press, a paper by Elmendorf *et al.*(12) appeared in which 24-hour, urinary excretions by 6 patients of isonicotinyl hydrazide after a single oral dose of 3.0 mg per kg (total dose = 140-200 mg) are reported to range from 47.8 to 70.7% of the dose (average =

61.0%). These excretion values, determined colorimetrically by the p-dimethylaminobenzaldehyde reaction(13) are almost twice as high as those reported above for 9 normal subjects (average excretion of Rimifon after 125 mg dose = 32% and after 192 mg dose = 33%) and correspond closely to the total excretion of isonicotinic acid plus Rimifon as determined by the cyanogen bromide-ammonia method (average total excretion of both 125 and 192 mg doses = 60%). This difference may be due to a) non-specificity of the p-dimethylaminobenzaldehyde reaction or b) a difference between tubercular and normal subjects. Although no Rimifon data have been obtained by the cyanogen bromide test on tubercular patients, the latter possibility appears unlikely in view of the similarity in excretion patterns of Marsilid by both types of subjects. Five male tubercular patients excreted approximately the same percentage of Marsilid in 24 hours as the above normal subjects (10-18%), the Marsilid in both groups being determined from the difference between assays by the cyanogen bromide method before and after permanganate treatment. Since isonicotinic acid, which is measured by direct assay with cyanogen bromide, does not react with p-dimethylaminobenzaldehyde, the differences in Rimifon excretions by the two assay methods can not be due to the isonicotinic acid present. It is possible that the hydrazine split from the Rimifon appears in the urine in another form which is measured by the p-dimethylaminobenzaldehyde reaction.

The Rimifon excretions by normal subjects as determined by the cyanogen bromide test have been confirmed microbiologically by the method of Tabenkin *et al.*(14), which measures Rimifon but not hydrazine or isonicotinic acid. Pooled, 24-hour urines from the test shown in Fig. 2 yielded microbiological assays for Rimifon, which were generally in close agreement with the chemical values.

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A Method of Positive Pressure Anesthesia for the Rat.* (19477)

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Herein described is a method of positive pressure anesthesia for the rat that has proved to be superior to those methods employing intubation and tracheotomy.

The apparatus consists of 2 Erlenmeyer flasks joined by rubber tubing to a positive pressure mask (Fig. 1). Flask A contains water and receives oxygen from a standard tank. The water serves to humidify the gas

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