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Distribution, metabolism and excretion of caprylo-and nicotino-hydroxamic acid

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DISTRIBUTION, METABOLISM AND EXCRETION OF CAPRYLO- AND NICOTINOHYDROXAMIC ACID

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Hydroxamic acid has been known to inhibit urease activity of gastrointestinal tract and decrease the level of blood ammonia. Using caprylo- and nicotinohydroxamic acid-[carbonyl-^{14}C] which are potent inhibitors of urease activity, distribution, metabolism and excretion of the compounds were studied after their oral administration to rats and guinea pigs.

Caprylohydroxamic acid was rapidly hydrolyzed by liver homogenate to caprylic acid and hydroxylamine, and hydroxamic acid was not detected in any tissues except in gastrointestinal tract 2 hr after its administration. Radioactivity derived from hydroxamic acid was found in the liver and heart in considerable amounts, but most of total radioactivity was excreted by expiration as CO_2 and several percentage was excreted in urine within 24 hr.

Nicotinohydroxamic acid was slowly decomposed by liver homogenate and a small amount of hydroxamic acid remained in the tissues 2 hr after its administration. Radioactivity derived from hydroxamic acid was observed considerably in the liver and kidney, and widely distributed in other tissues. Half of total radioactivity was excreted in urine and a few percentage as CO_2 within 24 hr. A part of the radioactivity was retained in the body and excreted gradually only in urine. Pattern of metabolites of nicotinohydroxamic acid in urine was similar to those of nicotinamide already reported.

The authors previously reported[2] that hydroxamic acids were strong inhibitors of urease activities originating in plants and micro-organisms. It was also demonstrated[3] that although hydroxamic acids characteristically strongly inhibited only the activity of urease, they showed practically no toxicity against mammalians. Furthermore, it was reported[4] that when, based on the information described above, a few hydroxamic acids were tested for possible use in medical treatment, they were found to be highly effective in preventing and treating the ammonemia of domestic animals, which occurs when urea is used as an additive of the feed for the animals. This effects was thought to have been caused by the characteristically strong inhibitory activity of the hydroxamic acids against urease existing in the gastrointestinal tract, and the resulting suppression of formation of ammonia from the urea.
At the same time, since the major cause of the hepatic coma often experienced by hepato-cirrhosis patients is considered to be due to the increase of concentration of blood ammonia, attention has been focused on hydroxamic acids as potential drugs of choice for the treatment[3,5]. Accordingly, it has been believed that clarification of the fate of hydroxamic acids <u>in vivo</u> would provide useful informations required to promote this group of chemicals to clinically useful drugs.

In order to achieve this objective, caprylo- and nicotinohydroxamic acids, which are the most potent inhibitors

of the urease activity of all the aliphatic and aromatic hydroxamic acids respectively, were chosen and their in vivo metabolic pathways were examined. Namely, these two hydroxamic acids labelled at their carbonyl carbons with ^{14}C were synthesized, and distribution and excretion of these compounds in rats and guinea pigs and metabolism in rats were examined after their oral administration to rats and guinea pigs. The results are reported in this paper.

DESCRIPTION OF EXPERIMENTS p.1565

^{14}C-Labelled Hydroxamic Acids

Caprylohydroxamic acid-carbonyl-^{14}C was synthesized from n-octanoic acid-1-^{14}C (250 μCi, 20 mCi/mmole, R.C.C.) according to the method of Inoue and co-worker[6]. When it was recrystallized from benzene, it showed m.p. 78-79°, and its radio-chemical homogeneity was confirmed by TLC (thin layer chromatography) and PPC (paper chromatography). The product was diluted with non-radio-active caprylohydroxamic acid, dissolved in ethanol, and further diluted with water to a final concentration of 1 μmole/ml (1% aqueous alcoholic solution), which was used for the experiments. The specific radio-activity of this product (diluted solid before dissolution)* was 1.77×10^6 cpm/mg by the gas flow counter method.

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*Translator's Note: Added by the translator.

Nicotinohydroxamic acid-carbonyl-^{14}C (m.p. 167°) was kindly donated by Eizai Co. Ltd.[7]. The radiochemical homogeneity was confirmed by TLC and PPC. The radio-active nicotinohydroxamic acid (16.3 μCi/mg) was diluted with non-radio-active nicotinohydroxamic acid synthesized by the method of Hacklby and associates[8], to 18.1., and used after dissolving in water (10 mg/ml). The product (diluted solid before dissolution) showed a specific radio-activity of 4.39×10^4 cpm/mg by the gas flow counter method and 3.48×10^5 cpm/mg by the liquid scintillation counter method.

Determination of Distribution in Internal Organs

As experimental animals, male Wister rats weighing between 100 and 200 g, and male Hartly guinea pigs weighing between 500 and 600 g, were used. When ^{14}C-labelled caprylohydroxamic acid was tested, the solution was orally administered with the aid of a stomach tube* (at 1.27 mg/Kg), and the animals were clubbed to death 1 hour or 24 hours after administration. During the periods of experiments, the animals were given free access to water and solid feeds (Nippon Kurea**). In the case of application of ^{14}C-labelled nicotinohydroxamic acid, the solution was orally administered with the aid of a stomach tube at 10 mg/Kg and the animals were

*Translator's Note: The authors use a German word "Sond(e)".
**Translator's Note: Transliterated.

sacrificed at 4 hours or 75 hours after administration by clubbing. The sacrificed animals were immediately subjected to bleeding, and their internal organs and glands were removed, washed with cold physiological saline to remove the blood, and the organs were weighed after drying by blotting with filter paper. The organs were homogenized in a blender after addition of a certain constant amount of water, and a portion of the blended mixture was sampled in a small dish, and its radio-activity was measured by a 2π gasflow counter (model JDC-303, manufactured by Nippon Musen Co.). The correction for the self-absorption of the test samples was performed by the internal standard method. The results of measurement were expressed by the average value (cpm/wet weight (g) of an organ) of 4 or 5 animals.

Measurement of ^{14}C in Breath

A ^{14}C-labelled hydroxamic acid solution was orally administered to rats, and the breathing of the animals was continuously passed through an aqueous monoethanolamine solution to collect CO_2 in the breathing at timed intervals. A 0.5 ml portion of the ethanolamine solution containing the CO_2 collected was added to 10 ml of a Bray solution[9], and the radio-activity was determined by means of a liquid scintillation counter (Model 3003 of Packard Co.) The calibration of quenching was done by the external standard line method.

Determination of ^{14}C in Urine and Feces

Rats and guinea pigs were kept in a urinary cage under free access to solid feeds (Nippon Kurea) and water, and the test solution containing each of ^{14}C-labelled hydroxamic acid was orally administered with the aid of a stomach tube. Urine and feces were collected at time intervals for a period of 2 weeks. The combined urine was subjected to centrifugation to remove insoluble matters, and 10 ml of a Bray solution[9] was added to 0.5 ml of the supernatant, and the radio-activity of the mixture was measured by a liquid scintillation counter. The content of ^{14}C in the feces was first checked on a portion of feces collected at a 48th hour of the administration. However, when the radio-activity in the specimen was measured by a 2π gasflow counter and also by the liquid scintillation counter method on a water extract and on an ethanol extract of the feces, there was no radio-activity detected. Therefore, no time-course measurement of the radio-activity in the feces was performed.

Quantitative Determination of Hydroxamic Acids

Based on the fact[2] that hydroxamic acids specifically strongly inhibit the urease activity, a method of quantative determination of hydroxamic acids in the organs and glands was invented. The relationship between the concentration of nicotinohydroxamic acid and the rate of inhibition of the urease activity is shown in figure 1.

The urease solution used for the experiment was prepared as follows. Namely, to powdered horse-beans (sword-bean), 5 volumes of a 0.1 M Tris-HCl-0.5 mM EDTA buffer solution (pH 7.4) was added, and the mixture was stirred for 30 minutes at 37° to extract the urease, and the mixture was centrifuged at 1000rpm for 15 minutes to remove insoluble matters. The crude urease solution (570 units/ml, 81.5 units/mg protein) was used. The hydroxamic acid was first dissolved in the same buffer solution as described above and used for extraction of urease, and 0.3 ml (containing 0.5-10 mμ moles) of the hydroxamic acid solution was diluted with 0.2 ml of the crude urease solution described above, and the mixture solution was incubated at 37° for 30 minutes. Residual urease activity was measured by the colorimetric method of van Slyke and co-worker[10]. The rate of inhibition was calculated using the activity of the urease solution containing no added hydroxamic acid as the standard.

The amount of hydroxamic acid in organs was determined by measuring the rate of inhibition of urease activity and referring to the line of correlation shown in figure 1. Namely, 4 to 6 ml of the same Tris-HCl-EDTA buffer solution as described above was added to 1 g wet weight of the organ, and the mixture was homogenized, and then heat-treated at 100° for 5 minutes. The mixture was centrifuged at 3000 rpm for 5 minutes and the supernatant was filtered through a piece of gauze. The rate of inhibition of the urease activity by 0.3 ml of this filtered supernatant was the basis of calculation of concentration

of the hydroxamic acid in the filtrate. When this analysis was performed, it was confirmed that the organs of the sacrificed animals did not contain urease or any other substances interfering with the assay. The extract solutions prepared from the organs of rats not administered with the hydroxamic acid, prepared in the same manner as described above for the organs of the treated rats, did not inhibit the urease activity. Furthermore, it was p.156 confirmed that the hydroxamic acid was not at all degraded by the heat-treatment in theneutral solution described above. The limit of this quantitative analysis of nicotinohydroxamic acid by the urease inhibition method is, as shown by figure 1, 5×10^{-7} = 0.5 mμ moles/ml. That is to say, the nicotinohydroxamic acid in the internal organs may be detected at a concentration of approximately 0.1 μg/ml.

Enzymatic Degradation of Hydroxamic Acid

To the liver of untreated healthy rat, 6 volumes of pH 8.0, 0.1 M Tris-HCl buffer was added, and the mixture was homogenized in a blender, and filtered through gauze. To 1.0 ml of the filtrate, 1.0 ml of a 1×10^{-2}M solution of hydroxamic acid was added and warmed at 38°C, and 0.5 ml of 50% trichloroacetic acid was added at 1 hour intervals and after standing at room temperature for 15 minutes, the mixture was centrifuged. The residual hydroxamic acid was measured by the method of Lipmann and associate[11] using 1.5 ml of the supernatant.

Identification and Qualitative Assay of Hydrolysis Products of Caprylohydroxamic Acid

To 1.2 g of an acetone powder of normal rat liver prepared in the usual manner, 13 ml of 0.1 M Tris-HCl buffer solution, pH 8.0, was added, and the acetone powder was extracted at 20° for 60 minutes. A supernatant of centrifugation of the mixture was used as the enzyme solution of the hydrolysis experiment. To 8.5 ml of the enzyme solution, 8.5 ml of a 1×10^{-2} M caprylohydroxamic acid solution was added. After the mixture was incubated at 38° for 60 minutes, the hydrolysis was quenched by addition of 0.6 ml of 3 N HCl. The fatty acid produced was determined as follows. The reaction solution was centrifuged and the supernatant was extracted three times with ether, and the ether layers were combined, evaporated and methylated with an ether solution of diazomethane. Ether was evaporated, the residue was dissolved in a small amount of acetone and the solution was analyzed by gas liquid chromatography (GLC). The GLC analysis was performed using a Shimazu TCD apparatus, model GC-2C, equipped with a 2.25 m long column packed with 5%-SE-30, using H_2 gas as a carrier and at a temperature of 100°C. The acid was analyzed qualitatively and quantitatively by comparing its retention time and the weight of the peak area cut out of the chromatogram with those of an authentic sample of methyl caprylate separately synthesized from caprylic acid.

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*Translator's Note: Probably at the injection port or possibly column but too low.

The hydroxylamine produced by the hydrolysis was analyzed as follows. The aqueous layer after the three extractions with ether was treated with trichloroacetic acid to remove the proteins, and pH of the residual solution was adjusted to 6.5 by addition of a dilute sodium hydroxide solution, and then oxidized with iodine. Nitrous acid produced was determined by the diazo method. The reaction conditions and concentration of each reagent solution employed for the diazo method were equivalent to those applied in the Feigl method[12] of qualitative analysis of hydroxylamine. Since the enzyme solutions contain a factor which partially interferes with the colorization, the enzyme solution that did not include added hydroxylamine was first subjected to the same reaction conditions and treatment procedures as the actual determination conditions of hydroxylamine, and then after additions of known amounts of hydroxylamine, the quantitative assays were performed. From these determination values and the blank test value a calibration line was drawn, and the actual determination was performed using the calibrated quantitation line.

Qualitative Analyses of Metabolites in Urine

A 100 mg/Kg quantity of ^{14}C-labelled nicotinohydroxamic acid was orally administered to male Wister rats by means of a stomach tube, and urine was collected for 6 hours. About 10 μl of the urine was spotted on Toyo No. 51 filter paper, and the spot was developed by a

two dimensional ascending method with n-butanol saturated with 3% ammonia water or* with n-butanol saturated with water. The filter paper after development was cut in 1 cm wide strips starting at the sample spot, and they were soaked in a Bray solution[9] overnight, and then the radio-activity was determined by a liquid scintillation counter. The urine collected before administration of the hydroxamic acid was used as a blank solution for the counting.

RESULTS

Distribution of ^{14}C in Internal Organs

In table I, distributions of radio-activity in rat organs after oral administration of ^{14}C-caprylo- and nicotinohydroxamic acid are shown.

a) Caprylohydroxamic Acid

The radioactivity distribution at 1 hour after administration of ^{14}C-caprylohydroxamic acid shows that the stomach content includes a large quantity of unabsorbed hydroxamic acid. However, the radio-activity is already widely distributed in all the organs, and the count per wet weight of each organ indicates that the organ distribution is in the decreasing order of lung, muscle, spleen, liver and kidney and the largest total count per organ is found in the liver. Comparison of this distribution with that at 24 hour after the administration shows that the count per weight decreases in the kidney, lung and spleen

*Translator's Note: Possibly "and then".

in the latter , but increases in the heart and liver, suggesting that accumulation of the ^{14}C-fragments occurs in the latter organs. Apart from the radio-activity determinations, a large quantity (200 mg/Kg) of non-radio-active caprylohydroxamic acid was administered, and after 2 hours, the undegraded hydroxamic acid itself was determined by the urease inhibition method, in each of the organs. In Considering the radio-activity distribution in each organ, shown in table I, after administration of the small quantity (1.27 mg/Kg) of ^{14}C-caprylohydroxamic acid, and also the specific radio-activity (1.77×10^6 cpm/mg) of the acid administered, if all the radio-activity counted in each organ was due to the undegraded hydroxamic acid, then the hdroxamic acid should have been detected because of the high sensitivity, 0.1 μg/ml, of the quantitative assay method. However, the caprylohydroxamic acid administered could not be detected in any organs other than in the gastrointestinal tract. This finding clearly demonstrates that the radio-activity detected in each organ was not due to caprylohydroxamic acid itself but rather due to its metabolites, probably some derivatives belonging to the fatty acid group.

b) Nicotinohydroxamic Acid

The radio-activity of ^{14}C-nicotinohydroxamic acid at 4 hours after oral administration is found to be widely distributed in every organ, but it is highest in the contents of the gastrointestinal organs and in the gastro-

intestinal organs themselves. It is also quite noticeable in the liver, kidney and spleen. The distribution of total count per organ was highest in the liver, followed by the kidney and testicle. At 75 hours after administration, about 70 to 90% of the radio-activity recorded at the 4 hour of administration was lost and at the same time, the different concentrations in different organs were averaged out. The count per organ was again the highest in the liver, followed by the kidney. There was no noticeable difference between the 4 hour radio-activity value and the 75 hour value in the heart and muscle. The authors determined distribution of the nicotinohydroxamic acid in every organ by the urease inhibition method. The quantitation limit (0.5 mμmole/ml; 0.07 μg/ml) is approximately the same as the precision limit of the radio-activity determination method, based on the specific radio-activity (4.4×10^4 cpm/mg) of nicotinohydroxamic acid. However, since the preliminary experiment showed that the majority of the hydroxamic acid was metabolized within a short period, a large amount (200 mg/Kg) of nicotinohydroxamic acid was orally administered, and the distribution of unchanged acid was determined after a relatively short period (2 hours). Contrary to caprylohydroxamic acid, which could not be detected at all, nicotinohydroxamic acid could be confirmed to be distributed in all the organs without being metabolized, although in minute quantities. Amounts of the unchanged hydroxamic acid in the organs were found to be relatively large in the muscle, liver, lung and kidney.

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Table II is the distribution of radio-activity in guinea pig organs after oral administration of ^{14}C-nicotinohydroxamic acid (10 mg/Kg) to two groups of four animals in each group. One group was sacrificed after 2 hours of administration for measurement of the radio-activity and the other group after 50 hours. After 2 hours, fairly large quantities of unabsorbed hydroxamic acid were found in the

contents of the stomach and intestines, and the radio-activity was considerably high in the liver and kidney, followed by the spleen and lung. In this case also, the radio-activity per organ was highest in the liver, and next in the kidney but very little in the other organs. The radio-activity distribution after 50 hours was highest in the kidney and liver, followed by the spleen and small intestine, while it was fairly constant in all the other organs. Comparison of the distribution values at 50 hour with those at 2 hour revealed that the activity in the heart increased slightly, those in the large intestine, spleen, brain, kidney and liver decreased slightly and those in other organs decreased very noticeably.

Detection of possible differences in the mode of distribution between different species of animals, rat and guinea pig, was attempted by comparison of table I and table II, but no noticeable difference could be found.

Excretion into Breath and Urine

a) Caprylohydroxamic Acid

Each of three male rats was orally administered with 218 μg (3.3×10^5 cpm) of ^{14}C-caprylohydroxamic acid, and excretion of the radio-activity into the breathing, urine and feces was determined at timed intervals, and average values of the rates of excretion against the amount administered are shown in figure 2 and table III. As shown in figure 2,

nearly 50% of the administered radio-activity was excreted as $^{14}CO_2$ in the breathing within 4 hours after administration, and about 70% within 24 hours. Thus the metabolism of this compound proceeds quite rapidly. The small rate of excretion into feces clearly indicates that ^{14}C-caprylohydroxamic acid almost completely disappears from the gastrointestinal tract by absorption and metabolic degradation. To sum up, a large portion of the radio-activity is excreted by breathing within 24 hours of administration and about 7% is excreted into the urine within the same period, and after the 2nd day, the amounts of excreted radio-activity become very small. Based on these results, the authors estimate that at least the fatty acid moiety of caprylohydroxamic acid is rapidly absorbed and introduced into the fatty acid metabolism pool, and the major portion of the pooled fatty acid quickly undergoes metabolism to CO_2, and the residual portion remains in various tissues as lipids.

b) Nicotinohydroxamic Acid p.1569

^{14}C-Nicotinohydroxamic acid was orally administered to 5 rats and 5 guinea pigs at either 10 mg/Kg or 553 μg/Kg, and the radio-activities in the breathing, urine and feces were determined at intervals. Figure 3 shows the average values of excretion rates from rats administered at 10 mg/Kg into their breathing. After 2 to 4 hours of administration, the excretion rate into the breathing reaches the peak value, lowers within 8 hours and after that only small amounts of excretion can be detected. After the

2nd day and later, there is practically no excretion observed and the total amount of excretion into the breathing is 2.85% of the total amount of administration. When the administration was done at 553 μg/Kg, the peak excretion could be observed at 2 hours, but the trend in the change of the excretion rate was nearly identical to that in the higher dosage. The total excretion in the breathing was about 1.3% of the total amount administered.

Figure 4 shows the average values of excretion rates from rats orally administered with ^{14}C-nicotinohydroxamic acid at 10 mg/Kg into the urine. About 17% is excreted in 2 hours after administration, and the rate decreases gradually during the 24 hours after administration, about 50% of the administered radio-activity being excreted within the first one day period. Later, the excretion rate continues to decrease for about 1 week, but even after 2 weeks there are still some small amounts of excretion detected. The total amount of excretion into urine in a 15 day period is between 70 and 75% of the total amount of administration. Again the same trend of the excretion pattern could be observed when 553 μg/Kg was administered. Since excretion into feces could not be detected within 48 hours of administration, no time-course determination of the excretion into feces was performed.

On the other hand, when excretion into the breathing of guinea pigs was determined, as in the case of administration to rats, the majority of the excretion in the form

of CO_2 took place within 8 hr., 4.8% (10 mg/Kg) and 1.5% (553 μg/Kg administration) of the administered nicotinohydroxamic acid being excreted within 2 days. In this case, excretion into the urine took place at rates of 26% (10 mg/Kg administration) and 22% (553 μg/Kg administration) within 2 hours, and the rates gradually decreased till the 8th hour, and slowly thereafter. However, even on the 15th day, the rates of excretion continued to be about 1% of the administered doses. The total amounts of excretion into the urine within 24 hours were 65% (10 mg/Kg) and 45% (553 μg/Kg), and those within 15 days were 85% (10 mg/Kg) and 75% (553 μg/Kg) of the administered doses. These results are nearly identical to those obtained by the administration to rats, indicating no difference due to the difference in the species of animals. To sum up the results described above, the excretion of nicotinohydroxamic acid as CO_2 into the breathing is 2.6% on an average, and that into the urine is on an average about 75%, and the course of the excretion forms a striking contrast to that of caprylohydroxamic acid.

In this experiment, carbonyl ^{14}C-nicotinohydroxamic acid was used as the labelled compound, and at present there is no known metabolic system for this compound to yield $^{14}CO_2$ other than that via the microbial ring opening reaction of the pyridine ring and the ensuing degradation reaction[13]. For this reason, the authors speculate that formation of CO_2 and excretion into breathing are also caused by the metabolic actions of intestinal microbial organisms.

The fact that about 80% of the administered nicotinohydroxamic acid was excreted into the urine and about 50% was found in the 1st day urine clearly demonstrates that the absorption and metabolism of the acid were quite fast. A portion of the administered acid was retained in the body and excreted slowly into the urine. In consideration of the known metablic route[13] of nicotinic acid, the administered nicotinohydroxamic acid appears first to undergo either hydrolysis or reduction to the amide derivative and then the products are incorporated into the in vivo nicotinic acid metabolism pool. When these occur, most of the excess amount of the nicotinic acid or its amide not incorporated into the metabolic pool is rapidly excreted into the urine as their metabolites but a portion may remain in the body possibly as structural components of various coenzymes.

Enzymatic Degradation of Hydroxamic Acid p.1570

In order to verify that hydroxamic acids indeed undergo enzymatic degradations in internal organs, caprylo-, nicotino- and benzohydroxamic acid were incubated with a rat liver homogenate, and amounts of residual hydroxamic acids were determined at timed intervals. As clearly shown in figure 5, about 70% of caprylohydroxamic acid degrades within 1 hour, and nearly completely in 3 hours. In comparison, quantities of remaining nicotinohydroxamic acid decreased more slowly, and about 50% degraded in 3 hours. Decrease of benzohydroxamic acid was extremely

slow, and about 80% remained after 3 hours.

Furthermore, in order to see if the enzymatic degradation is specifically an enzymatic hydrolysis, the following experiments were carried out. Namely, using caprylohydroxamic acid, which is the fastest degrading, as the substrate, qualitative and quantitative analyses of the products of the enzymatic reaction with an extract of rat liver acetone powder were performed.

When the rate of degradation of caprylohydroxamic acid became 68%, the reaction was quenched by acidification with hydrochloric acid, and the reaction solution was extracted with ether. The ether extractive fraction was methylated with diazomethane, and the resulting fatty acid methyl ester was qualitatively and quantitatively analyzed. When the ester was subjected to an GLC analysis, the peak retention time corresponded well with that of separately synthesized methyl ester of caprylic acid, and further, the methyl ester was quantitatively assayed by weighing the peak area shown in the chart (Table IV). As control reactions, the enzyme solution employed and caprylohydroxamic acid were individually treated in the same manner as described above and subjected to the same GLC analysis, but no peak corresponding to the acid methyl ester could be detected. Using the aqueous solution, from which protein that remained in it after the ether extraction was removed, the hydroxylamine in the residual solution was assayed by the diazo method[12]. The stoichiometry of the enzymatic degradation listed in table IV proved that caprylohydroxamic acid is hydrolyzed

with practically no accompanying side reaction.

Examination of Urinary Metabolites Derived from Nicotino-Hydroxamic Acid

Metabolites of ^{14}C-nicotinohydroxamic acid excreted in rat urine were analyzed by PPC and TLC. Since metabolites of nicotinic acid amide excreted by mice were examined previously by Bonarita and associates[14] and by Chaykin and co-workers[15], the authors' results were compared with these published results.

When a urine specimen was subjected to PPC with two different solvent systems, n-BuOH-3% NH_3 and n-BuOH-H_2O, five radio-active spots could be identified. In table V, the Rf values of these spots and relative intensities of radio-activity of each are compared with the results of the experiment reported by Bonarita et al[14] and Chaykin et al[15]. Based on the comparison of the Rf values, it was estimated that the major metabolites were N-methylnicotinamide-4-one (about 50%) and N-methylnicotinamide-6-one (about 17%). N-Methylnicotinuric acid and N-methylnicotinamide have identical Rf values on the paper developed with the two different solvents employed in the reports already mentioned. Therefore, although the authors also detected a radio-active (17% and 28% depending on the solvent system) spot at the Rf value corresponding to these compounds, whether the radio-active spot was due to either one of them or both at an unknown mixture ratio could not be determined. As minute components of the metabolite mixture, two spots apparently due to

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nicotinamide (2-3%) and its N-oxide (2-6%) could be detected at close to the Rf values cited in the reports. However, there was no detectable radio-activity at the Rf of nicotinohydroxamic acid on the paper strips developed with both solvent mixtures.

When a urine specimen was evaporated to dryness, extracted with hot acetone and the extract subjected to chromatography on an ion-exchange resin column prepared with Dowex-50 (Na^+ type), about 25% of the radio-activity contained in the urine specimen was eluted as a sharp single peak. When UV absorption of the peak fraction was measured in a neutral medium, the spectrum showed a maximal absorption band at 260 mμ, which agreed very well with the spectrum of N-methylnicotinamide-4-one isolated by Wu-Chang and co-worker[16] as a metabolic product of nicotinic acid by rat. They also reported[16] that the absorption maximum 260 mμ of this compound in a neutral medium shifted to 240 mμ in an acidic medium. The authors' spectra of the major eluate fraction in both neutral and acidic media and the shift of the maximum absorption band by change of pH of the medium completely agreed with the data reported in the reference[16].

Nicotinamide-N-oxide was isolated from the urine specimen by cocrystallization method, and the specific radio-activity of this compound showed that the compound was responsible for about 2.6% of the total radio-activity in the urine.

DISCUSSION

It has been attempted to protect animals from the toxicity of ammonia by suppressing formation of ammonia from urea by means of an inhibition of urease activity in the gastrointestinal tract, which can be achieved by dosing the animals with hydroxamic acids through the oral route. In order to assure the safety and efficacy of hydroxamic acids as drug substances, it is extremely important to clarify the fate of hydroxamic acids in the body. To meet this objective, the authors examined the distribution of hydroxamic acids in the animal body, the route of their metabolism and the process of their excretion by orally administering hydroxamic acids to rats and guinea pigs. As the test compounds, caprylohydroxamic acid was chosen as a representative of fatty acid hydroxamic acids, because of its low toxicity and of its great capacity for inhibiting urease activity, although the rate of inhibition is small [2,17], and nicotinohydroxamic acid as a representative of aromatic hydroxamic acid because of its high water solubility and low toxicity coupled with the lack of difficulty encountered in oral administration due toits acceptable taste, and also because of its strong activity in the inhibition of urease activity[2]. The present experiments enabled us to clarify modes of absorption from the gastrointestinal tract, and distribution and metablolism in the body and excretion from the body, by tracing the radio-activity of the carbonyl-^{14}C derivatives of these hydroxamic acids. The urease activity in the gastro-

intestinal tract is high in the lower small intestine, in the caecum and in the upper colon. The inhibitory action of hydroxamic acids on the urease in the gastrointestinal tract may come into effect via two routes; one is after directly passing through the gastrointestinal tract and reaching the lower part of the tract, and the other is after once being absorbed through the tract into the body fluid and then reaching the lower part of the tract. Judging from the rates of metabolism and excretion, it appears that the inhibitory actions demonstrated by the test compounds on the intestinal urease are demonstrated only for a short limited period after being orally administered, and that consequently, only a small portion of the administered acid demonstrates the inhibitory activity.

Based on the results (Table IV) of identification and quantitation of the products of hydrolysis of the absorbed caprylohydroxamic acid by the enzyme extracted from an acetone powder of liver and also on the fact that there was no detectable amount of caprylohydroxamic acid remaining intact in the internal organs, it appears that the caprylohydroxamic acid absorbed undergoes a rapid hydrolysis in the liver. The enzymes participating in the hydrolysis are probably protease, peptidase, amidase, esterase and other enzymes which may be characterized by a rather wide range of substrate specificity. The tracing of the radio-activity of the carbonyl carbon-labelled caprylohydroxamic acid has shown that about 40% and almost all of the total activity are excreted

into the breathing of animals within 4 hours and within 24 hours, respectively, and these results clearly demonstrate that the caprylic acid produced by the hydrolysis has been incorporated into the fatty acid metabloism system.

On the other hand, in the case of nicotinohydroxamic acid, the authors could confirm that it was gradually degraded by the liver homogenate employed. The experiments, however, proved only that it disappeared, not clarifying the mode of reactions involved. Hirsch and co-worker[18] confirmed that nicotinohydroxamic acid was reduced to an amide by an enzyme(s) localized in liver mitochondria, but whether or not this reaction was the major route of the metabolism remains unknown. Only 2 to 3% of the radio-activity of the labelled carbonyl carbon of nicotinohydroxamic acid could be detected in the breathing but since ring opening of nicotinic acid has been demonstrated only by micro-organisms, the minute quantity of radio-activity detected in breathing may bave been caused by the microbial metabolism of organisms existing in the gastrointestinal tract. When nicotinohydroxamic acid is absorbed, it is hydrolyzed to nicotinic acid or its amide, and brought into the nicotinic acid metabolism pool, but any amount of the acid or amide in excess of retention capacity of the pool is probably rapidly excreted into the urine. A portion of that is widely distributed among various internal organs and excreted into the urine as metabolites in a long span of time. The latter type of excretion was probably caused by another role of the absorbed

nicotinic acid, namely that of converting itself into a component of vitamines. As such, it plays a variety of physiological roles, rotates while undergoing metabolism and finally appears in the urine. The metabolic product mixture that appeared p.157
in the urine immediately after administration was qualitatively analyzed by PPC in comaprison with the metabolic products of nicotinamide by mice and rats[14,15], and it was estimated that the mixture consisted of N-methylnicotinamide-4-one as the chief component, N-methylnicotinamide-6-one, either one or both of N-methylnicotinuric acid and N-methylnicotinamide, and nicotinamide-N-oxide.

In the past, studies on the metabolic products of hydroxamic acids have been carried out on only several compounds. Namely, McIsaac and co-worker[19] reported that benzohydroxamic acid was excreted into the urine as hippuric acid, when it was metabolized by rabbit and sheep, and they[19] also found that salicylohydroxamic acid was reduced to an amide by man, rabbit, rat and mouse. Bernheim[20] reported that hydroxamic acids derived from various fatty acids were hydrolyzed by a certain kind of lipase found in the liver, and Fishbein and co-worker[21] proved that when a large quantity of acetohydroxamic acid was administered to a mouse recovery from the urine of the unchanged hydroxamic acid was 65-70% and conversion to acetamide was 10-20% of the administered hydroxamic acid. In the authors' experiment, both caprylo- and nicotinohydroxamic acid administered were completely metabolised, and no unchanged hydroxamic acids could be

detected in the urine as demonstrated by identification of the radio-active spots on PPC strips or by detection of a substance with an inhibitory activity against urease. Most of the metabolites in urine after administration of nicotinohydroxamic acid were identified to be amides. Regarding the exact pathways of the general metabolism of hydroxamic acids, whether it is a direct reduction to the corresponding acid amides, a route via amide rearrangement or an amide formation after hydrolysis has not been clarified, mainly because there has not been a sufficient amount of evidence to prove the in vivo reaction sequence. Furthermore, since the metabolic pathway varies considerably depending on the acyl moiety of different hydroxamic acids, it is difficult to draw any generalized conclusion.

The authors did not examine the in vivo fate of hydroxylamine also produced by the hydrolysis. However, Reimann[22] already reported that hydroxylamine administered to mouse was completely metabolized and not detected in the urine. Colter and coworker[23] proved that hemoglobin catalytically decomposed hydroxylamine into ammonia and molecular nitrogen under unaerobic conditions. Therefore, the authors believe that the hydroxylamine produced as a hydrolysate of hydroxamic acids was also further decomposed through this same route.

In this paper, the authors present a novel method of quantitative analysis of hydroxamic acids based on the strong activities of hydroxamic acids in the inhibition of the

urease activity. Against the sensitivity of the detection limit of 0.5 μmole of the colorimetric method using ferric ion devised by Lipmann and co-worker[11], the present method had a detection limit of 0.5 mμmole for nicotino- and caprylo-hydroxamic acid, or about 1,000 times as sensitive as the Lipmann method. The fault of the present method has been found in the variability of sensitivity depending on the variation of the R group of hydroxamic acid (R-CONHOH). However, if a standard inhibition curve of each hydroxamic acid is first prepared, then the method is probably usable for qualitative determination of thioester, acyl phosphoric acid and other minute components of which the in vivo identification has been very difficult. In this report, the authors determined the in vivo distribution of administered nicotinohydroxamic acid (Table I). When the results of the analysis of the hydroxamic acid obtained by the urease activity inhibition method and the results of the radio-activity counting method for one and the same internal organ were compared, it was found that the large portion of the radio-activity was caused by metabolites of the hydroxamic acid and practically no unchanged hydroxamic acid existed in organs other than in the gastrointestinal tract. Such comparisons on each organ also indicated an amount of apparent metabolism in each organ as well as the degree of movement of the hydroxamic acid and its metabolites from one organ to another. Since the conditions of the experiments varied for each determination and also since the time course changes of these analyses

on each organ were not measured, no further precise comparisons could be made. Nevertheless, it was found that the radio-activity was high in the spleen, liver and kidney but the content of hydroxamic acids in these organs was remarkably low. Also found was the fact that in the muscle tissues, considerably large amounts of the unchanged hydroxamic acids were contained. This is to say, the activity of the enzyme(s) that hydrolyzed hydroxamic acid is high in the spleen, liver and kidney and it is low in the muscle tissues. This new quantitative assay method is therefore considered to be highly effective in analyzing in vivo distribution of unreacted hydroxamic acids themselves, which cannot be determined by the distribution of radio-activity per se.

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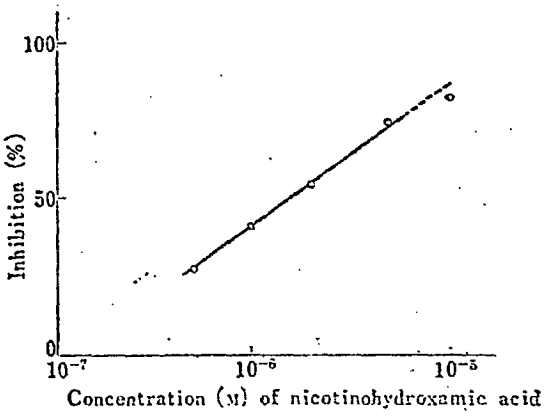

Fig. 1. Determination of Nicotinohydroxamic Acid by Its Inhibition of Urease Activity

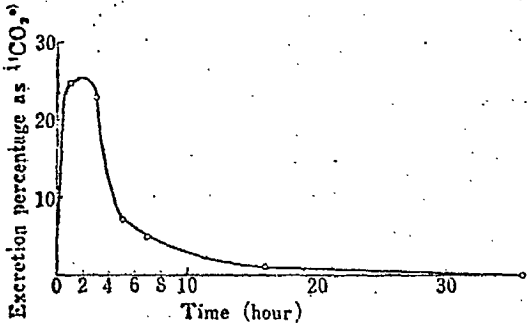

Fig. 2. Excretion of Radioactivity by Breathing after Oral Administration of Caprylohydroxamic Acid-1-^{14}C in Rats

a) average percentage of excretion as $^{14}CO_2$ of three male rats

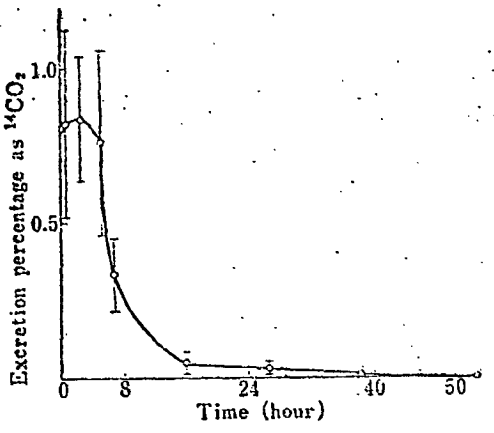

Fig. 3. Excretion of Radioactivity by Breathing after Oral Administration of ^{14}C-Nicotinohydroxamic Acid (10 mg/kg) in Rats

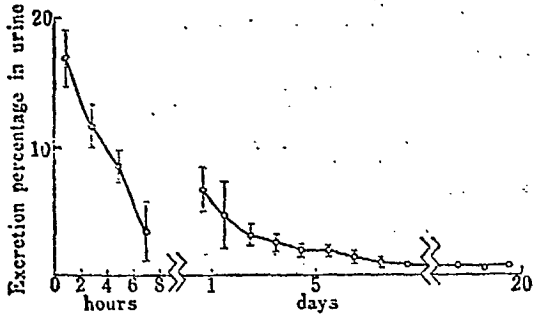

Fig. 4. Excretion of Radioactivity in Urine of Rats after Oral Administration of ^{14}C-Nicotinohydroxamic Acid (10 mg/kg)

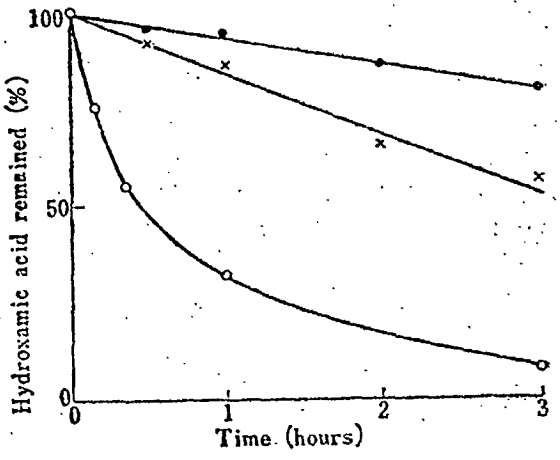

Fig. 5. Enzymatic Decrease of Hydroxamic Acid by Rat Liver Homogenate

●—● :benzohydroxamic acid
×—× :nicotinohydroxamic acid
○—○ :caprylohydroxamic acid

TABLE I. Distribution of Radioactivity in Rat Organs after Oral Administration of ^{14}C-Hydroxamic Acids

	Caprylo-HXA-1-^{14}C (1.27 mg/kg)[a]				Nicotino-HXA-^{14}C (10 mg/kg)				Nicotino-HXA (200 mg/kg) 2 hr	
	1 hr (3 rats)		24 hr (2 rats)		4 hr (4 rats)		75 hr (4 rats)			
	cpm/g	Total cpm	cpm/g	Total cpm	cpm/g ±S	Total cpm	cpm/g ±S	Total cpm	HXA μg/g[b]	HXA Total μg
Brain	—	—	—	—	4090± 650	8280	2160± 971	3840	0.91	1.8
Lung	3330	4250	624	811	5420± 519	12500	1920± 82	7670	7.4	26
Heart	1380	1240	5460	4370	4330± 305	6060	4490± 700	6620	4.3	7.2
Spleen	2650	1640	1350	877	11400± 1420	9700	2960± 214	2670	1.4	1.4
Liver	2600	34100	5780	73300	26200± 1130	470000	5150±1940	98100	2.6	51
Kidney	1420	4300	298	715	19600± 2080	81700	6190±2260	25400	2.6	20
Testicle	—	—	—	—	5260± 526	32600	617± 148	4100	2.6	7.9
Muscle	3320	—	741	—	3920± 740	—	3130±1040	—	17	—
Stomach	7660	11500	3770	6410	11800± 4360	—	3240± 198	—	62	185
Stomach content	35300	—	9890	—	33700± 2030	—	576± 189	—	—	—
Intestine	1530	—	2300	—	21300± 4720	—	1130± 321	—	165	1275
Intestine content	5850	—	3270	—	14900±12000	—	1100± 165	—	—	—
Colon	—	—	—	—	8190± 2730	—	658± 469	—	2.2	13
Colon content	—	—	—	—	13000± 1410	—	123± 255	—	—	—
Blood	1360	—	815	—	3500± 140	—	625± 165	—	—	—

a) cpm values shown are the average of results of two or three animals.
b) Hydroxamic acid was determined by its inhibitory power of urease activity as described in Experimental section.

TABLE II. Distribution of Radioactivity in Guinea Pig Organs after Oral Administration of ^{14}C-Nicotinohydroxamic Acid (10 mg/kg)

Tissue	2 hr (4 rats)		50 hr (4 rats)	
	cpm/g±S	Total cpm	cpm/g±S	Total cpm
Brain	3630± 247	14000	2690± 667	10900
Lung	10500± 576	54200	4770±1300	21700
Heart	3970± 617	7930	5810± 691	11200
Spleen	11700±1520	10800	9510±1630	8560
Liver	26000±2200	977000	16100±2850	500000
Kidney	25700±3100	159000	17200±3620	85400
Testicle	5920± 675	44500	2770± 165	20700
Muscle	7050± 444	—	3100± 115	—
Stomach	12200±1040	—	2820± 420	—
Stomach content	88100±4710	—	3670±1300	—
Intestine	23000±2580	—	8520±3080	—
Intestine content	28500±5980	—	4680± 782	—
Colon	4270±1350	—	4500± 880	—
Colon content	4820±1080	—	535± 204	—
Blood	3570± 263	—	732± 82	—

TABLE III. Excretion of Radioactivity in Urine and Feces after Oral Administration of Caprylohydroxamic Acid-1-^{11}C in Rats

	Day	1	2	3	4
Excretion %	urine	6.9	0.4	0.0	0.0
	feces	0.6	0.3	0.0	0.0

TABLE IV. Stoichiometry of Enzymatic Hydrolysis of Caprylohydroxamic Acid

Caprylohydroxamic acid decreased (μ moles) 204	Caprylic acid formed (μ moles) 179	Hydroxylamine formed (μ moles) 171
$FeCl_3$ method[11]	gaschromatography of its ester	diazo-method[12]

TABLE V. Proposed Metabolites of Nicotinohydroxamic Acid in Rat

Metabolites	BuOH–3% NH_3 *Rf*			BuOH–H_2O *Rf*		
	Ref.[13]	Sample	(%)	Ref.[14]	Sample	(%)
N-Methylnicotinuric acid N-Methylnicotinamide	0.04	0.067	(17)	0.045	0.045	(28)
Nicotinamide-N-oxide	0.30	0.28	(6)	0.23	0.31	(2)
N-Methylnicotinamide-4-one	0.43	0.43	(52)	0.28	0.38	(45)
N-Methylnicotinamide-6-one	0.51	0.51	(15)	0.35	0.44	(18)
Nicotinamide	—	0.65	(3)	0.60	0.63	(2)
Nicotinohydroxamic acid	0.21[a]	—	(0)	0.55[a]	—	(0)

a) Nicotinohydroxamic acid-carbonyl-^{11}C was dissolved into urine of control rats and developed under the same condition.