cyanoacetic acid were absent from the urine and symptoms of β-aminopropionitrile toxicity were not observed. The nontoxic compounds were those not containing an amino acyl group.

Although the mode of action of β-aminopropionitrile has not been defined, previous studies (Follis and Tousimis, 1958; Levene and Gross, 1959; Smiley *et al.*, 1962) have clearly associated increased levels of soluble collagen with lathyrism. Attempts to demonstrate an *in vitro* effect of β-aminopropionitrile on collagen fibril formation (Levene and Gross, 1959) were not successful. Whether the *in vivo* effects of β-aminopropionitrile involve a direct interference with conversion of soluble into native collagen or an indirect effect is the main question now under consideration. The emphasis which the present studies place upon the necessity for enzymatically liberating free β-aminopropionitrile[2] before these acyl derivatives can produce symptoms of osteolathyrism suggests further studies with radioactive β-aminopropionitrile.[3] An incorporation of the radioactivity into soluble collagen would support the view of a direct effect of β-aminopropionitrile, possibly by chemical binding through its amino group. Strong evidence that β-aminopropionitrile is directly bound to collagen *in vivo* has in fact been very recently obtained by Levene (1962) and by Stalder and Stegemann (1962) with nonradioactive material.

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[2] Enzymes capable of cleaving the γ-glutamyl group from α-aminonitriles and from 4-hydroxymethyl-phenylhydrazine have recently been encountered in human blood serum (Szewczuk, 1959; Szewczuk and Orlowski, 1960) and in the edible mushroom, *Agaricus bisporus* (Levenberg, 1961), respectively.

[3] Earlier studies (Garbutt, 1958) with β-aminopropionitrile of relatively low specific activity indicated that only traces of the radioactivity were incorporated into rat tissues. Similar low incorporation of the radioactivity of C^{14}-labeled aminoacetonitrile was observed by Ponsetti *et al.* (1956).

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Metabolic Fate of Kynurenic Acid-C^{14} Intraperitoneally Administered to Animals*

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Received August 23, 1962

Kynurenic acid-2- or 3-C^{14} was intraperitoneally administered to the rat, mouse, hamster, rabbit, guinea pig, cat, and dog in a dose of 0.6 μmole per 100 g body weight. With all the animals tested, more than 90% of the dose given was excreted in the urine during the first 24 hours, with little or no radioactivity in expired CO_2 or carcass. Most of the radioactivity of the first-day urine was present as unchanged kynurenic acid (80–100%). The excretion of small amounts of quinaldic acid (1.3–4.8%) and quinaldylglycine (0.3–1.8%) was also detected.

In contrast to the recent accumulation of experimental evidence indicating a rapid metabolism of kynurenic acid to L-glutamic acid by tryptophan-adapted cells of *Pseudomonas fluorescens* (Hayaishi *et al.*, 1959, 1961; Behrman and Tanaka, 1959; Horibata *et al.*, 1961), no such active utilization of kynurenic acid by animals has been reported. Instead, earlier papers (Kotake and Ichihara, 1931; Correll *et al.*, 1938) showed that kynurenic acid subcutaneously administered to rabbits and dogs was nearly quantitatively recovered as such within 24 hours from the urine and bile. However, in view of the fact that the daily output of kynurenic acid in the urine of an untreated animal (Brown and Price, 1956) is far less than the dose those investigators applied, the possibility still existed that an appreciable metabolism of kynurenic acid could be detected if the latter were administered to the animal in a dose close to its physiological output. The present paper deals with the results of such experiments where a small dose of kynurenic acid-2- or 3-C^{14} was administered to several species of animals and the

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* This work was supported in part by research grants from the National Institutes of Health (C-4222), the Rockefeller Foundation, and the Jane Coffin Childs Memorial Fund for Medical Research.

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distribution of radioactivity in expired CO_2, urine, feces, and carcass was determined.

METHODS AND MATERIALS

Kynurenic Acid-2- or 3-C^{14}.—Kynurenic acid-C^{14} was prepared from DL-tryptophan-C^{14} as described in the previous communication (Hayaishi *et al.*, 1961). DL-Tryptophan-2′-C^{14}, 0.53 mC/mmole, from Tracerlab Inc., and DL-tryptophan-3′-C^{14}, 5.6 mC/mmole, from Nuclear-Chicago Corporation, were used as starting materials. Both products were shown to be pure by paper chromatography with several different solvent systems.

Experimental Animals.—Two Wistar strain rats, a DD strain mouse, and five other different, wild strain species as listed in Table I were used. All the animals were female. Small animals were chosen in order to minimize the urinary volume. The animals were fed a stock grain diet, with vegetables in some cases, and during urine collection they were kept in stainless steel metabolism cages fitted with glass funnels.

Administration of Kynurenic Acid-C^{14}.—Each animal was given a single, intraperitoneal injection of a neutralized solution of kynurenic acid, 0.6 μmole per 100 g body weight. A total of one to three million cpm, as measured with Nuclear-Chicago Corporation thin-window gas-flow counter, was administered to each animal. In one experiment (rat no. 2 in Table I) 100 μmoles of cold kynurenic acid was injected intraperitoneally immediately after the radioactive kynurenic acid was administered.

Expired CO_2.—With one rat, kept in an all-glass metabolism cage, the expired CO_2 was collected at hourly intervals for 10 hours after the administration of kynurenic acid-C^{14} and the radioactivity was measured as $BaCO_3$ (Weinhouse and Friedmann, 1951).

Carcass.—The carcass of one mouse together with its viscera and intestinal contents was acetone-ether dried (Gholson *et al.*, 1958) and analyzed for C^{14} content by the method of Van Slyke *et al.* (1951).

Urine and Feces.—The urine samples were collected daily and held under toluene at 4°. For radioactivity measurements the urine was filtered and plated and the radioactivity counted. The results were corrected for background radiation and extrapolated to infinite thinness. The undiluted urine samples were then stored frozen until needed for fractionation (see below). The feces, collected as separately from the urine as possible, were analyzed for radioactivity either by water extraction or as acetone-ether dried material (Gholson *et al.*, 1958). The results of both analyses agreed within experimental error.

Fractionation of Urine.—The 24-hour urine or a portion of it was fractionated by a procedure essentially the same as that described by Kaihara (1960). The fractionation consisted of successive use of columns of Dowex 50 (H^+) (8% cross-linkage, 200–400 mesh) and Dowex 2 (acetate) (8% cross-linkage, 200–400 mesh) (Roy and Price, 1959). The size of the column and the volumes of solvents were adjusted to suit the size of the sample (Roy and Price, 1959; Kaihara, 1960). Of the six fractions listed in Table II, the first three were obtained from the Dowex 50 column and the rest from the Dowex 2 column. The radioactivity was measured for each fraction, and identification of the radioactive compounds was carried out by several different methods.

Paper Chromatography.—Ascending and descending paper chromatography was used for the identification of the various quinoline compounds (Roy and Price, 1959; Kaihara, 1960). Whatman no. 1 paper was used with the following solvent systems: methanol–*n*-butanol–benzene–water (2:1:1:1) containing 1% acetic acid or NH_4OH (Price and Dodge, 1956), *n*-butanol–acetic acid–water (4:1:5), and 80% *n*-propanol. The samples obtained by concentration of the urinary fractions mentioned above, or sometimes by subsequent extraction of the concentrate with ethanol, were chromatographed with authentic compounds as references. The paper was examined under ultraviolet light, and the radioactivity on paper strips was located and measured either with an automatic recorder actigraph, Nuclear-Chicago Corporation, or by cutting the strip into 1-cm sections which were attached to aluminum planchets and counted with a gas-flow counter.

RESULTS

Recovery of Radioactivity from Animals After Administration of Kynurenic Acid-C^{14}.—It is apparent from Table I that with all the experimental animals tested more than 90% of the kynurenic acid administered appeared in the first 24-hour urine specimen. Practically no radioactivity was found in expired CO_2, but under the conditions employed exact measurement could not be made if the radioactivity present was less than 0.2% per hour, mainly owing to a high degree of dilution. Since the carcass of the mouse was found to contain little radioactivity, no other samples were analyzed.

TABLE I

PER CENT RECOVERY OF RADIOACTIVITY FROM ANIMALS AFTER INTRAPERITONEAL ADMINISTRATION OF KYNURENIC ACID-C^{14}

The dose of kynurenic acid given to animals was 0.6 μmole per 100 g body weight except for rat no. 2, to which 100 μmoles were administered.

		C^{14} Recovered				
		Urine		Feces		
Animal	Body Weight (g)	1st day (%)	2nd day (%)	1st day (%)	Expired CO_2 (%)	Carcass (%)
Rat no. 1	150	93	0.5	3.9	<2[a]	
Rat no. 2	160	92	0.6	3.1		
Mouse	25	97		3.0		2[b]
Hamster	40	94	2.0	4.9		
Rabbit	305	99	0.5	0.2		
Guinea pig	300	90		4.6		
Cat	1800	96	1.2	1.9		
Dog	4000	90		1.8		

[a] For initial 10 hours (see text). [b] 24 hours after administration.

Distribution of Radioactivity in Various Urine Fractions.—When the first 24-hour urine specimen was subjected to fractionation on a column with Dowex 50 (H^+), most of the radioactivity was found to be associated with the fraction which passed through the column with a large volume of water (Fig. 1). This material was further fractionated on a column of Dowex 2 (acetate) to yield fractions 4, 5, and 6. Table II represents two typical protocols of experiments on the radioactivity distribution among the six fractions thus obtained. The close similarity in distribution with a rat and a rabbit can be seen from the table. The almost quantitative recovery of radioactivity after completion of fractionation, shown as "Total (1 to 6)" in the table, is significant, since this indicates that no

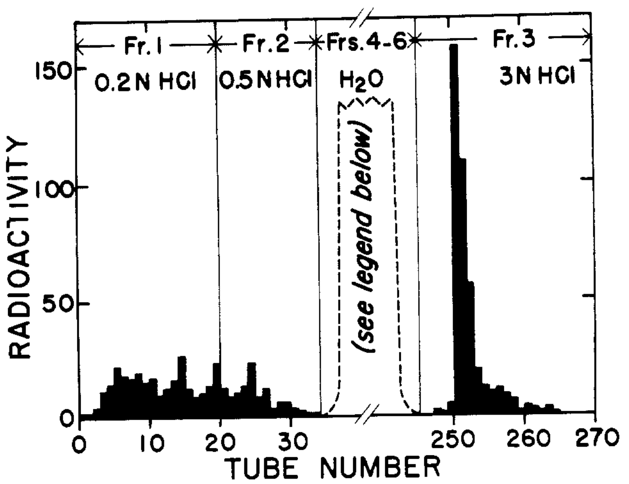

FIG. 1.—Elution diagram of radioactivity in the urine of rat no. 1 from Dowex 50 (H^+) column. The radioactivity shown is corrected for background radiation. Five ml of urine was diluted with sufficient water and 1.0 N HCl to bring the volume to 25 ml with an acid concentration of 0.2 N. The mixture was applied to a column 1.12 cm in diameter and 5 cm long. The elution was carried out as indicated in the figure. Each tube contained 3 ml, 0.05 ml of which was plated, and the radioactivity counted. Radioactivity found in combined aqueous eluates (broken line) amounted to more than 90% of that applied to the column; the combined eluates were further treated with a Dowex 2 (acetate) column to obtain fractions 4 to 6.

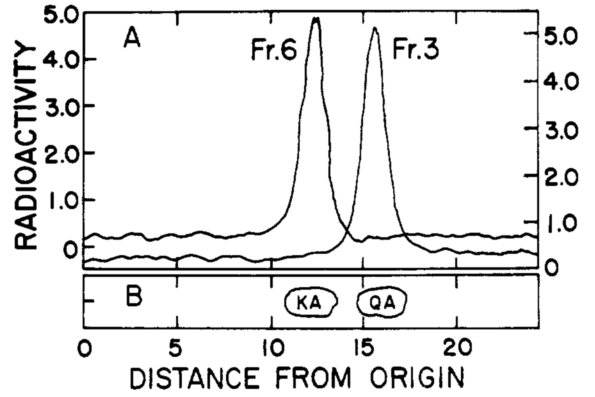

FIG. 2.—Identification of kynurenic acid-C^{14} in fraction 6 and quinaldic acid-C^{14} in fraction 3 from the urine of rat no. 1 by paper chromatography on Whatman no. 1 paper with 80% *n*-propanol. In part *A* are shown radioactivity recordings of chromatograms for fractions 6 and 3. In part *B* the spots are tracings of fluorescence of authentic kynurenic acid (KA) and quinaldic acid (QA), which were run on the same chromatogram. The actigraph was used with a slit 0.318 cm in width and a scanning speed of 20.3 cm/hour. With both fractions 6 and 3 the total radioactivity of the sample applied was approximately 2000 cpm. Radioactivity scales in part *A* are actigraph chart units, and a full scale (5.0) roughly corresponds to 300 cpm under the conditions employed.

other major metabolites were formed in addition to those found in the six fractions. The table also shows that approximately 90% of the original radioactivity in the urine applied was recovered in fraction 6.

TABLE II

DISTRIBUTION OF RADIOACTIVITY IN VARIOUS FRACTIONS OF THE FIRST 24-HOUR URINE SAMPLE

Fractions 1 to 3 were obtained from Dowex 50 (H^+) column and fractions 4 to 6 from Dowex 2 (acetate) column (see text).

Fraction No.	Eluted by	Urine of Rat No. 1 (cpm)	Urine of Rat No. 1 (%)	Urine of Rabbit (cpm)	Urine of Rabbit (%)
1	0.2 N HCl	14,500	2.4	18,000	2.8
2	0.5 N HCl	2,000	0.3	3,000	0.5
3	3 N HCl	22,000	3.7	20,000	3.1
4	2 N acetic acid	1,400	0.2	8,000	1.2
5	4 N acetic acid	6,000	1.0	3,500	0.5
6	7 N acetic acid	539,000	89.8	570,900	87.7
Total (1 to 6)		584,900	97.5	623,400	95.9
Urine applied		600,000	100	650,000	100

Identification of Radioactive Kynurenic Acid in Fraction 6.—A tracing of the actigram for radioactive compounds found in fraction 6 from the urine of rat no. 1 is shown in Figure 2. The fraction contained only one radioactive compound; its R_F value coincided exactly with that of authentic kynurenic acid. This was true also with solvent systems other than that shown in Figure 2. To confirm the identity of the radioactive compound in fraction 6 with kynurenic acid the following experiments were conducted. An aliquot of fraction 6 was applied to a column of Dowex 1 (formate) and the elution was carried out with formic acid as previously described (Hayaishi *et al.*, 1961). The radioactivity applied was quantitatively recovered from those tubes in which authentic kynurenic acid should appear. The radioactive eluate was concentrated *in vacuo* and subsequently the neutralized solution was subjected to crystallization with nonradioactive kynurenic acid added as carrier. Two recrystallizations from acid were followed by two from boiling water. The results obtained, shown in Table III, indicate a constant specific activity throughout the course of recrystallization.

TABLE III

RECRYSTALLIZATION OF KYNURENIC ACID-C^{14} RECOVERED FROM FRACTION 6 OF RAT URINE

51.8 mg of carrier kynurenic acid was used per 5.0 ml of the test solution, corresponding to a total of 22,000 cpm. Recrystallization with acid was performed by bringing the *p*H of the solution below 2.0 with 2 N HCl. The precipitated crystals were dissolved in 0.1 N KOH for the measurement of radioactivity and kynurenic acid concentration. The latter was calculated from the absorbancy of the solution at 333 mμ at *p*H 6.5. After the second crystallization with acid the crystals were dissolved in 100 ml of boiling water for further recrystallization.

Recrystallization	Crystal (cpm/mg)	Mother Liquor (cpm/mg)
Original solution	4,370	
1st crystallization	4,190	—[a]
2nd crystallization	4,320	—[a]
3rd crystallization	4,380	4,320
4th crystallization	4,480	4,590

[a] No measurement could be made because less than 1% of the material was present in the mother liquor.

Identification of Radioactive Quinaldic Acid in Fractions 3 and 4.—Fraction 3 from the urine of rat no. 1 was found to give only one radioactive area on paper chromatograms with several different solvent systems. A typical example of such an actigram is shown in Figure 2, which suggests the identity of the compound with quinaldic acid. Fraction 4 also gave the same result. Kaihara (1960) had described in a similar fractionation experiment that quinaldic acid accounted for 100% of the radioactivity found in fractions 3 and

4. Nevertheless, in view of the large excess in the present experiment of the radioactivity found in fraction 6 over that found in fraction 3 (Table II), special care had to be taken to accomplish a complete separation of quinaldic acid from kynurenic acid. Thus, in column chromatography on Dowex 50 (Fig. 1) the elution of fraction 3 with 3 N HCl was started only after 20 tubes or more were collected beyond the one which no longer showed radioactivity, assuring that kynurenic acid had been completely eluted. In Figure 2, the actigram of quinaldic acid found in fraction 3 is shown in comparison with that of kynurenic acid found in fraction 6 in order to illustrate the good separation of two compounds attained.

Identification of Radioactive Quinaldylglycine in Fraction 5.—Fraction 5 from the rat urine gave a radioactive area on the chromatogram with *n*-butanol–acetic acid–water which ran faster than quinaldic acid (Fig. 3*A*). The R_F value calculated led to the suspicion of the presence of quinaldylglycine (Roy and Price, 1959; Kaihara, 1960). In order to prove this, the radioactive area was cut out and eluted with 0.2% NH_4OH. The eluate was evaporated to dryness *in vacuo* and hydrolyzed with 2 N HCl in a sealed tube at 110° for 6 hours. After removal of HCl the material was chromatographed on paper with the same solvent system as above. No radioactivity was found in the area ahead of quinaldic acid, and, instead, the presence of radioactive quinaldic acid was revealed (Fig. 3*B*). A ninhydrin-positive spot corresponding to the R_F value of glycine also appeared on the chromatogram after acid hydrolysis of the suspected "quinaldylglycine" material obtained from the urine of rat no. 2 to which approximately 100 times as much kynurenic acid had been given as to rat no. 1.

Identification of Radioactive Constituents in Fractions 1 and 2.—According to Kaihara's experiment (1960), a portion of the quinaldic acid present appears in fractions 1 and 3, and it accounts for 88% of the radioactivity of fraction 1 and 100% of that of fraction 2. However, this was not the case in our experiment. Thus, fractions 1 and 2 from the urine of rat no. 1 were combined and evaporated *in vacuo* to a thick paste. The material was dissolved in water to approximately 5,000 cpm per ml. The solution was fractionated on a column of Dowex 1 (formate) (Takahashi *et al.*, 1956) with nonradioactive quinaldic acid added as carrier. As shown in Table IV, it is apparent that the radioactivity found in fractions 1 and 2 is to be attributed to a compound or compounds which, like kynurenic acid, is held more tightly to the resin than quinaldic acid. The material eluted by washing the column with 12 N formic acid was identified with kynurenic acid by means of paper chromatography with *n*-butanol–acetic acid–water.

TABLE IV

RECHROMATOGRAPHY OF COMBINED FRACTIONS 1 AND 2 OF RAT URINE ON A DOWEX 1 (FORMATE) COLUMN WITH CARRIER QUINALDIC ACID

To 1 ml of combined concentrates from fractions 1 and 2 was added 32 mg of authentic quinaldic acid. The solution was applied to a column 1.12 cm in diameter and 2 cm long of Dowex 1 (formate) and the gradient elution was carried out with 6 N formic acid. One-ml fractions were collected, and the absorbancy at 322 mμ at *p*H 6.5 due to quinaldic acid was measured with each fraction. After all the material absorbing at 322 mμ had appeared, the column was washed with 20 ml of 12 N formic acid.

Fraction	Quinaldic Acid (mg)	Radioactivity (cpm)
Original solution	32.0	2,500[a]
Fractions before appearance of absorption at 322 mμ, combined	0	0
Fractions showing absorption at 322 mμ, combined	28.8	100
Eluate with 12 N formic acid, combined		4,780

[a] No extrapolation of the radioactivity to infinite thinness was made.

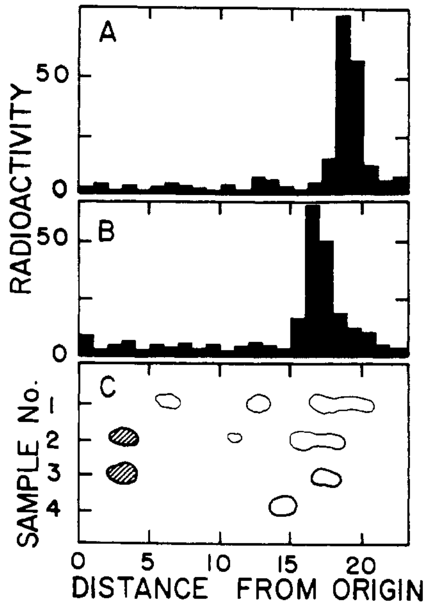

FIG. 3.—Identification of quinaldylglycine-C^{14} in fraction 5 from the urine of rat no. 2 by paper chromatography on Whatman no. 1 paper with *n*-butanol–acetic acid–water. Parts *A* and *B* show the radioactivity distribution among 1-cm sections from paper strips no. 1 and no. 2, respectively, of part *C*. In part *C*, no. 1 is a sample from fraction 5 before acid hydrolysis; no. 2 is a sample from fraction 5 after hydrolysis in 2 N HCl at 110° for 6 hours; no. 3 is authentic quinaldic acid *plus* glycine; no. 4 is authentic kynurenic acid. The shaded spots in part *C* are tracings of ninhydrin color and those not shaded are fluorescent areas. The radioactivity shown is corrected for background counts.

Identification of Radioactive Constituents in Urine of Various Animals.—Radioactive constituents of urinary fractions from animals other than the rat were examined as described above. Identifications were made mostly by paper chromatography. The results, similar to those described for rat urine, suggest that the radioactivity of fractions 1, 2, and 6 can be attributed to kynurenic acid, that of fractions 3 and 4 to quinaldic acid, and that of fraction 5 to quinaldylglycine. In Table V is summarized the per cent distribution of radioactivity among these three compounds in the first 24-hour urine samples of various animals. The similar distribution found with a variety of experimental animals should be noted.

DISCUSSION

The conversion of kynurenic acid to quinaldic acid in humans and rats was reported by Takahashi *et al.* (1956), but since oral administration was employed in their experiments, the conversion could have arisen also from bacterial action on kynurenic acid in the intestine. In fact, it was well known that oral administration always results in a much lower recovery of kynurenic acid from the urine when compared to subcutaneous injection (Correll *et al.*, 1938; Takahashi *et al.*, 1956), and this was supposed to be attributable

TABLE V

PER CENT DISTRIBUTION OF RADIOACTIVITY AMONG QUINOLINE COMPOUNDS IN THE FIRST 24-HOUR URINE SAMPLE

Animal	Kynurenic Acid (%)	Quinaldic Acid (%)	Quinaldylglycine (%)	Total (%)
Rat no. 1	92.5	4.0	1.0	97.5
Rat no. 2		6.0	5.5	
Mouse	99.8	4.8	1.8	106.4
Hamster	89.5	3.6	0.4	93.5
Rabbit	91.0	4.2	0.6	95.8
Guinea pig	95.8	1.5	1.0	98.3
Cat	79.8	1.3	0.3	81.4
Dog	97.2	2.0	0.5	99.7

to the action of intestinal bacteria (Correll *et al.*, 1938). In order to minimize the complexity resulting from the possible involvement of bacterial action, intraperitoneal administration of kynurenic acid was employed in the present experiment. Although intraperitoneal injection does not insure noninvolvement of bacteria, since a portion of the kynurenic acid injected in a large dose was reported to appear in the bile (Kotake and Ichihara, 1931; Correll *et al.*, 1938), the experimental data presented above (Table V) probably reflect an ability of animal tissue to convert a small part of the kynurenic acid given into quinaldic acid and perhaps subsequently into quinaldylglycine. The excretion of quinaldylglycine in rat urine after the ingestion of quinaldic acid was reported by Kaihara (1960).

The finding that most of the kynurenic acid-C^{14} was excreted as such into the urine within 24 hours after administration (Tables I and V) is consistent with the earlier reports in which much larger doses of kynurenic acid were administered. These results indicate a relative inertness of kynurenic acid over a wide range of dosage. The metabolic inertness of kynurenic acid, even when given in a small dose, in several different species of animals is of particular interest in view of the fact that some of these animals differ distinctly in their modes of urinary excretion of kynurenic acid and other tryptophan metabolites before and after the administration of tryptophan (Kotake and Ichihara, 1931; Brown and Price, 1956).

Only recently Kaihara and Price (1962) reported that when 10.7 μmoles of kynurenic acid-carboxyl-C^{14} was administered to a rabbit 88.9% of radioactivity was recovered from the first 48-hour urine sample, of which 99.0% was found to be unchanged kynurenic acid, a result consistent with that of the present experiment. A similar result was reported also with a cat (Kaihara and Price, 1961), although after oral administration a much lower recovery of kynurenic acid from the urine was noted with excretion of a large amount of quinaldylglycyltaurine. The detection of quinaldylglycyltaurine was not studied in the present experiment because of the limited amount of material.

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