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The Uptake of ^{14}C-Labeled Hydroxyanthranilic Acid and Enantiomers of Tryptophan, Kynurenine, and Hydroxykynurenine in Human Blood[1] (38959)

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During studies of tryptophan metabolite metabolism in humans with scleroderma (1), the observed differences in metabolism of the various compounds into $^{14}CO_2$ posed the question of whether the differences were due to a difference in rate of absorption of the D- vs L-isomers of the components, or the ability of the host enzyme systems to utilize the two isomers. Work with the rat by Berg (2) indicated that there was no difference in the growth rate of rats fed the D- or L-isomers of tryptophan. Metabolism studies with rats, have shown that the rat utilized the D- or L-isomer of tryptophan with equal ability as far as conversion of labeled tryptophan into $^{14}CO_2$ and the various metabolites was concerned (3). Some workers have claimed that D-isomers are not transported thru the intestinal wall and that therefore this component cannot be metabolized by the animal (4). Aroskar and Berg (5) have shown that in the rat the D-isomer of tryptophan was absorbed at half the rate of absorption of the L-isomer. This study was conducted to determine whether reliable metabolism studies can be conducted in humans with labeled DL-mixtures of the amino acids, and whether tryptophan metabolic products are indeed bound to serum proteins.

Materials and Methods. In vivo *patient studies.* In the *in vivo* studies, patients were given tracer doses of D- or L-tryptophan, D- or L-kynurenine, or D- or L-3-hydroxykynurenine. The patient information and dosage schedule of labeled and unlabeled compounds administered are presented in Table I.

All patients were volunteers who, when informed of the experimental nature of the procedure, gave their informed consent. For a period of 5 days prior to and during the study period, the patients were on a controlled tryptophan diet containing an estimated 0.7–1.0 g per day. All forms of drug therapy were avoided during the study period, and all studies were done with patients in the resting state. All labeled doses were given orally in gelatin capsules 1 hr after breakfast.

The commercially available DL-[7a-^{14}C]-tryptophan used here was checked for purity by chromatography and autoradiography (6). The DL-[^{14}C]kynurenine-keto and the DL-[^{14}C]3-hydroxy-kynurenine-keto were synthesized in our laboratories (7, 8). The DL-[7a-^{14}C]tryptophan, DL-[^{14}C]kynurenine-keto, and DL-[^{14}C]3-hydroxy-kynurenine-keto were resolved into the D- and L-isomers by a paper powder column technique (6). The specific activities of the labeled compounds were determined by combustion to CO_2 and analysis in Ballentine gas counting tubes (9).

A series of 10 ml heparinized blood samples were drawn from each patient at 0.0, 0.5, 2, 4, 8, 12, and 24 hr. After centrifugation, the plasma was drawn off and stored frozen in small vials until analyzed.

A 50-μl sample of each plasma was dried in a Van Slyke combustion tube and analyzed for ^{14}C by the gas analysis method (9). The protein in the plasma was analyzed for ^{14}C by a precipitation technique previously published (10).

In vitro *serum uptake experiments.* Pooled human serum from a clinical chemistry laboratory was preserved by adding (0.05 ml/10 ml of serum) a solution containing 10,000 units of penicillin and 10,000 mCg of streptomycin/ml. The serum was divided into 10 ml quantities in sterile vials and stored frozen. For an incubation study, a vial of serum was thawed and the contents added to a 25-ml screw-top Erlenmeyer flask containing a weighed quantity of

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TABLE I. PATIENT DATA AND COMPOUNDS ADMINISTERED.[a]

Patient no.	A	B-1	B-2	C-1	C-2	C-3	D
Age	60	45	46	44	45	46	40
Labeled compound	L-KYN-K	L-KYN-K	H-L-KYN-K	D-KYN-K	H-D-KYN-K	L-TRY-7a	D-TRY-7a
(mg)	28.38	28.44	20.73	31.49	17.84	112.5	24.26
(μci)	25.40	25.46	23.33	28.18	20.66	10.62	49.46
L-Tryptophan (g)						2.0	
D-Tryptophan (g)							1.0

[a] All patients were female Caucasians who had scleroderma.

The abbreviations used in the table are: L-KYN-K, L-[^{14}C]Kynurenine-Keto; H-L-KYN-K, L-[^{14}C]-Hydroxy-Kynurenine-Keto; D-KYN-K, D-[^{14}C]Kynurenine-Keto; H-D-KYN-K, D-[^{14}C]Hydroxy-Kynurenine-Keto; L-TRY-7a, L-[7a-^{14}C]Tryptophan and D-TRY-7a, D-[7a-^{14}C]Tryptophan.

carbon-14 labeled material. The solution was stirred on a magnetic stirrer for 5 min and then five zero time samples taken for analysis. Three (50λ) samples were immediately put into Van Slyke carbon combustion tubes, which were placed in a hot water bath for a period of 5 min prior to placing them in a vacuum oven for drying. These samples were combusted to $^{14}CO_2$ to obtain total carbon-14 activity per milliliter of solution. Two (100λ) samples were taken for protein precipitation to determine percentage of activity bound to protein. All samples taken for protein precipitation were placed in pointed Van Slyke centrifuge-combustion tubes containing sodium tungstate, which precipitated the proteins immediately and stopped activity. The Erlenmeyer flask containing the serum and labeled material was shaken on a Dubnoff shaker and incubated at 37°. Duplicate protein analysis samples were taken at times 0.5, 2, 4, 8, 12, 24, and 48 hr. The tungstic acid protein precipitation procedure was adopted from previous work (10). In some studies, samples were also taken for protein electrophoresis at zero time, 8, 24, and 48 hr. These electrophoretic migration patterns were then autoradiographed (6). The Millipore Phoroslide System, which utilized 0.075 ion strength barbital buffer set at pH 8.6 was used for the electrophoresis migrations of the serums. A Beckman (model RB Analytrol) recording densitometer fitted with a microzone (slide) adaptor was used to read the stained electrophoresis slides and the autoradiograph films. The compounds studied were L-[^{14}C]kynurenine-keto, D-[^{14}C]kynurenine-keto, D-[7a-^{14}C]tryptophan, L-[7a-^{14}C]tryptophan, and [^{14}C]3-hydroxyanthranilic acid carboxyl (11).

To study temperature effects on plasma binding ability, plasma and L-kynurenine were placed in a water bath at 0.5° and allowed to cool to that temperature. The plasma was added to the flask containing the kynurenine and allowed to mix for 5 min. This was called zero time. Samples were taken at times zero, 28, 65, 87, and 121 min at which times the temperatures were 0.5, 10, 19, 28, and 37°, respectively. The 50 λ samples taken for total ^{14}C analyses were immediately dried, and analyzed for carbon-14. The 100λ samples taken (at the above bath temperatures) for bound ^{14}C analyses were immediately precipitated and the precipitate analyzed for carbon-14.

Results. Table II shows the results of the analyses of the blood samples taken at intervals during the patient studies. The plasma radioactivity levels reached maximum height at 2–4 hr in all those patients receiving L-isomers of tryptophan, kynurenine, or 3-hydroxykynurenine, and gave data similar to that of previous studies when a 2-g load of L-tryptophan was given with a tracer dose of DL-[2-^{14}C]tryptophan (12).

TABLE II. ANALYSIS OF PLASMA FROM SCLERODERMA PATIENTS RECEIVING CARBON-14 LABELED TRYPTOPHAN METABOLITES.

Patient	Compound	Dose (μCi)	Fraction of plasma	0.5 hr	2 hr	4 hr	8 hr	12 hr	24 hr
A	L-[^{14}C]Kynurenine-Keto	25.4	Bound ^{14}C (dpm/ml)	100.9	357.5	190.7	131.7	93.9	82.5
			Total ^{14}C (dpm/ml)	353.6	2208.6	1006.0	422.4	298.6	250.6
			Percent ^{14}C bound	28.5	16.2	19.0	31.2	31.4	32.9
B-1	L-[^{14}C]Kynurenine-Keto	25.46	Bound ^{14}C (dpm/ml)	53.0	97.0	109.0	110.0	119.0	35.0
			Total ^{14}C (dpm/ml)	233.0	712.0	512.0	480.0	234.0	74.0
			Percent ^{14}C bound	22.7	13.6	21.3	22.9	50.8	47.3
B-2	L-[^{14}C]Hydroxy-Kynurenine-Keto	23.33	Bound ^{14}C (dpm/ml)	14.6	61.9	154.5	81.6	50.3	28.5
			Total ^{14}C (dpm/ml)	113.9	247.2	809.3	416.5	247.4	85.1
			Percent C^{14} bound	12.8	25.0	19.1	19.6	20.3	33.4
C-1	D-[^{14}C]Kynurenine-Keto	28.18	Bound ^{14}C (dpm/ml)	3.35	23.7	33.8	80.7	49.8	29.9
			Total ^{14}C (dpm/ml)	7.0	35.0	70.5	282.2	144.5	35.5
			Percent ^{14}C bound	47.9	67.6	47.9	28.6	34.5	84.1
C-2	D-[^{14}C]Hydroxy-Kynurenine-Keto	20.66	Bound ^{14}C (dpm/ml)	6.1	13.0	16.7	32.4	8.3	5.2
			Total ^{14}C (dpm/ml)	9.7	73.0	138.8	187.1	109.5	15.1
			Percent ^{14}C bound	62.9	17.7	12.0	17.3	7.5	34 1
C-3	L-[7a-^{14}C]-Tryptophan	10.62	Bound ^{14}C (dpm/ml)	25.5	218.1	496.5	517.3	516.7	444.5
			Total ^{14}C (dpm/ml)	196.3	808.7	743.2	649.3	578.7	488.1
			Percent ^{14}C bound	13.0	27.0	66.8	79.7	89.3	91.1
D	D-[7a-^{14}C]-Tryptophan	49.46	Bound ^{14}C (dpm/ml)	1.4	12.0	173.2	610.4	857.0	975.0
			Total ^{14}C (dpm/ml)	20.1	65.3	893.7	1947.0	1452.4	1169.2
			Percent ^{14}C bound	7.0	18.4	19.4	31.4	59.0	83.4

In contrast, the peak plasma radioactivity levels in those subjects who received D-isomers of [7a-^{14}C]tryptophan, [^{14}C]kynurenine-keto, or [^{14}C]3-hydroxykynurenine-keto were delayed until 8 hr. This delay may be a function of the rate of absorption from the gut (13, 3). It was noteworthy that the actual activity bound in the plasma protein of the patient given D-[7a-^{14}C]tryptophan was still increasing at 24 hr. This may signify the strong binding of D-tryptophan or the conversion of the D-tryptophan into a component that was securely bound to plasma protein.

Table III shows the values of the analyses of serum samples removed from the flasks during the *in vitro* incubation studies. The labeled D- and L-isomers of tryptophan and kynurenine were readily bound to the serum proteins, as indicated by the initial binding of the amino acid at the zero time point and the lack of an increase in amino acid bound as the incubation progressed. On the other hand, 3-hydroxyanthranilic acid was very poorly bound at zero time, but as the incubation progressed, the percentages of the ^{14}C activity of the 3-hydroxyanthranilic acid that were bound increased.

Autoradiographs of the electrophoretic migration patterns showed that the D- and L-tryptophan were bound to the protein in three main sites—the albumin, beta globulin, and gamma globulin—with equal amounts of activity present in all three sites. The zero time autoradiographic pattern of the 3-hydroxyanthranilic acid run showed that most of the ^{14}C activity (unbound) migrated in front of the albumin fraction which had taken up a very small percentage of the total activity present. As the serum incubation progressed, the unbound ^{14}C fraction, which migrated in front of the electrophoresis pattern, decreased and the ^{14}C activity in the protein fractions increased. The serum proteins labeled were the albumin, alpha globulin and beta globulin fractions. There was very little uptake of ^{14}C label in the gamma globulin fractions in the 3-hydroxyanthranilic acid incubation study.

Table IV shows the effect of temperature on L-[^{14}C]kynurenine-keto binding in serum.

TABLE III. ANALYSIS OF PLASMA INCUBATED WITH CARBON-14 LABELED TRYPTOPHAN METABOLITES.

Compound	Fraction of plasma	0.0 hr	0.5 hr	2 hr	4 hr	8 hr	12 hr	24 hr	48 hr
L-[7a-^{14}C] Tryptophan	Bound ^{14}C (dpm/ml)	155154	147885	146562	149964	148984	147978	157126	161852
	Total ^{14}C (dpm/ml)	776460	776460	776460	776460	776460	776460	776460	776460
	Percent ^{14}C bound	19.98	19.05	18.88	19.31	19.19	19.06	20.24	20.84
L-[^{14}C]-Kynurenine-Keto	Bound ^{14}C (dpm/ml)	19911	18855	18911	19103	19680	20818	22693	27510
	Total ^{14}C (dpm/ml)	152720	152720	152720	152720	152720	152720	152720	152720
	Percent ^{14}C bound	13.04	12.35	12.38	12.51	12.89	13.63	14.86	18.01
D-[7a-^{14}C]-Tryptophan	Bound ^{14}C (dpm/ml)	84576	82962	85504	85411	82645	87219	95545	101382
	Total ^{14}C (dpm/ml)	588616	588616	588616	588616	588616	588616	588616	588616
	Percent ^{14}C bound	14.37	14.09	14.53	14.51	14.04	14.82	16.23	17.22
D-[^{14}C]-Kynurenine Keto	Bound ^{14}C (dpm/ml)	35941	34507	29810	30642	32668	33268	40469	50185
	Total ^{14}C (dpm/ml)	244464	244464	244464	244464	244464	244464	244464	244464
	Percent ^{14}C bound	14.70	14.12	12.20	12.53	13.36	13.61	16.55	20.53
[^{14}C]3-Hydroxy-Anthranilic Acid Carboxyl	Bound ^{14}C (dpm/ml)	10049	20173	116823	251885	337174	358612	371441	361930
	Total ^{14}C (dpm/ml)	468988	468988	468988	468988	468988	468988	468988	468988
	Percent ^{14}C bound	2.14	4.30	24.91	53.71	71.89	76.46	79.20	77.17

TABLE IV. EFFECT OF TEMPERATURE ON L-[^{14}C]KYNURENINE-KETO BINDING IN SERUM.

Temperature °	0.5	10	19	28	37
Time (min)	0.0	28	65	87	121
Bound ^{14}C (dpm/ml)	42880	39070	36770	36750	34880
Total ^{14}C (dpm/ml)	201988	201988	201988	201988	201988
Percent ^{14}C bound	21.23	19.34	18.20	18.19	17.27

The amount of ^{14}C activity bound by the serum did not increase as the temperature was raised from 0.5° to 37° over an incubation time of 2 hr.

Discussion. Previous studies in animals suggested that some amino acid D-isomers were not actively transported by rat intestine (13, 3). With the observed delay in absorption, it was thought that intestinal flora may play a role in the metabolism of the D-form isomers. In the present study a delay was observed in the appearance of the patients breath $^{14}CO_2$ (reported previously) (1), when the labeled D-isomer of tryptophan was given orally, and this delay was also reflected in the ^{14}C content of the patients urine samples, which showed the largest quantity of the ^{14}C-activity was present in the 6–12 hr collection period. Some of this activity may be D-kynurenine formed by enzymes in the intestinal mucosa. Yamamoto *et al.* (14) have shown that the rabbit intestinal mucosa contains an oxygenase capable of converting D-tryptophan into D-kynurenine. However, if D-kynurenine was formed in appreciable amounts from the D-tryptophan given to man in the present study, there should have been more similarities in the total amount of ^{14}C activity and free ^{14}C activity present in the plasma after ^{14}C labeled D-tryptophan or D-kynurenine administration. That the D-isomers are bound to plasma protein was shown in the plasma incubation studies, and illustrates that the D-isomers can change the metabolism patterns of the L-isomers in the human. The long 8-hr delay in the peaking of the ^{14}C activity in blood after administration of the D-isomers suggests a slow absorption of these materials from the gut, but may also indicate involvement of the intestinal wall in the scleroderma patient concerned. When the D-isomer of tryptophan was absorbed, it was apparently firmly bound to plasma protein in increasing quantities during the 8–24 hr period. The early appearance of ^{14}C in the plasma in the patients receiving the L-isomers suggests that they were rapidly

absorbed from the gut. Unbound ^{14}C activity in the plasma was cleared at a faster rate than the bound ^{14}C activity resulting in an increasing percentage of the total ^{14}C activity in the bound form (15). These observations suggest that when conducting amino acid studies in humans, the single L-isomer should be used because the D-isomer can possibly occupy metabolic sites normally taken by the L-isomers.

The use of DL-tryptophan in humans for studies of blood or urine constituents is open to considerable difficulty in interpretation. The present work demonstrates that the D-isomers are transported into the bloodstream at a slower rate than the L-isomers, presumably because of poor absorption. In addition, D-tryptophan (or D-tryptophan metabolites) are bound to plasma proteins firmly and in significant quantities.

In the *in vitro* incubation studies the 3-hydroxyanthranilic acid apparently was not initially bound in large quantities to plasma proteins as the intact compound. However, as the incubation period progressed, the hydroxyanthranilic acid was metabolized either to a straight chain compound (16) or a form (17), which was readily bound to the plasma protein. Though, the chemical nature of this component was not determined, the maximum rate of production of this component and its binding apparently occurred during the 2–4 hr period of incubation, when 28.8% of the activity was bound.

The serum incubation studies showed that D-tryptophan was bound to serum protein at a lower percentage than the L-isomer. In the *in vivo* studies, we found that the L-isomer total ^{14}C activity present in the serum proteins peaked at 2 hr while the D-isomer total ^{14}C activity peaked at 8 hr. The ^{14}C activity bound in terms of L-tryptophan plateaued during the 8–12 hr period, while the bound ^{14}C activity in the D-tryptophan patients' serum was still increasing. The isolation of labeled D-tryptophan from urine samples from the patient given D-tryptophan showed that 6–12 hr after the ^{14}C labeled dose was given, 36% of the ^{14}C dose (1) or 62% of the ^{14}C activity present in the 6–12 hr urine was present as D-tryptophan (1). This would indicate that a high level of intact D-tryptophan was present in the bloodstream during the period (8–12 hr) of increased binding of activity. During the incubation study, the fact that initially only a set quantity of D-[^{14}C]tryptophan activity was taken up by the protein lends credence to the theory that the subsequent additional quantity bound was possibly metabolites of D-tryptophan. The level of free tryptophan in the blood, when L-tryptophan was given orally would be dependent on a number of factors: such as the binding of the tryptophan products, kynurenine, or hydroxykynurenine, which have amino acid structures and could compete with L-tryptophan for the binding sites on the albumin molecules of the plasma. In addition, the plasma levels of the free compound depend on the levels of activity of the tryptophan pyrrolase enzyme which converts the tryptophan into the kynurenine pathway products.

Summary. Labeled enantiomers of tryptophan, kynurenine, and hydroxykynurenine were given to five patients with scleroderma. Blood samples were drawn periodically over a 24-hr period. When the D-isomers were given, peak blood levels of radioactivity did not appear until 8 hr, whereas with the natural L-isomers peak levels were reached in 2 hr Considerable radioactivity was found firmly bound to the precipitated plasma proteins from both the L- and D-isomers. Incubation studies confirmed the binding of the D-isomers to the serum proteins. These experiments show that the use of pure labeled L-isomers is required, when studying the metabolism of amino acids in man, and that compounds other than L-tryptophan are bound to serum proteins in significant quantities.

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