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THE ABSORPTION OF TRITIUM-LABELLED PYRIDOXINE HYDROCHLORIDE IN THE RAT

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Until recently it was only possible to study th eabsorption of pyridoxine indirectly by measuring the urinary excretion of pyridoxine and its metabolites following relatively large oral doses. The initial studies of Scudi, Unna & Antopol (1940) and Scudi, Koones & Keresztesy (1940) in man, the dog and the rat, were made using a colorimetic method for estimating pyridoxine and its metabolites. Although this method fails to estimate 4-pyridoxic acid, now known to be the principal urinary metabolite of pyridoxine in man (Reddy, Reynolds & Price, 1958), these workers did show that pyridoxine was excreted in the urine very soon after it was ingested, and they therefore concluded that absorption must be exceedingly rapid.

Recently pyridoxine hydrochloride labelled with tritium has become available, and it is now possible to measure small amounts of this substance with accuracy. In this paper we report the results of studies of absorption of tritiated pyridoxine in the rat. Observations were made on the rates and sites of absorption of varying doses; the capacity of the small intestine of the rat to absorb increasing amounts of pyridoxine was also studied.

METHODS

Experimental animals. Black and white rats weighing between 200 and 250 g were used throughout the experiments. Most of the observations were made on intact animals. A few studies were also made in a group of five animals in which between 50 and 70 % of the distal small intestine had been removed. These animals were prepared by the operative technique described by Booth, Evans, Menzies & Street (1959), and the experiments were carried out one month later when the animals had fully recovered and were in good health.

[^3H]*pyridoxine hydrochloride.* Commercial pyridoxine HCl prepared by British Drug Houses was labelled with tritium by the Radiochemical Centre, Amersham. The resulting material had a specific activity of 1·6 mc/mg and was 97 % pure by isotope dilution assay.

Radioactive test doses, containing between 0·05 and 5·0 mg of pyridoxine HCl, were fed to the animals. These doses were prepared by dilution of the labelled material and the addition of suitable amounts of unlabelled pyridoxine. Doses were usually adjusted so that 1 ml. contained the required amount of pyridoxine and 1 μc of radioactivity.

Electrophoresis of pyridoxine HCl and 4-pyridoxic acid. Pyridoxine HCl was subjected to paper electrophoresis at 120 V for 6–8 hr, with an acetate buffer (pH 5·1) as described by Siliprandi, Siliprandi & Lis (1954). When electrophoresis of a mixture of 4-pyridoxic acid and pyridoxine HCl was carried out, a carbonate buffer at pH 11·1 was used. Electrophoretic strips were first examined under ultra-violet light, and the pyridoxine band was identified by its fluorescence. They were then stained with diazotized *p*-aminoacetophenone. The bands were identified by comparison with a parallel run of each compound.

Measurement of radioactivity. All radioactive samples were counted in a Packard Tricarb liquid scintillation counter. The liquid scintillator used consisted of 2-5,diphenyloxazole 2 g/l. and 1,4-bis(2,5-diphenyloxazolyl)benzene 0·025 g/l. dissolved in a mixture of equal volumes of toluene and ethanol. Fluid specimens were assayed by mixing 0·25 ml. samples with 8 ml. of liquid scintillator. Internal standards were used throughout and were prepared from 0·25 ml. samples of 1 in 20 dilution of the test dose in the specimen to be assayed. To determine the distribution of radioactivity in electrophoretic strips the paper was cut into 0·5 cm. lengths, each of which was counted directly in 8 ml. of the liquid scintillator.

Experimental procedure

Urinary excretion of radioactivity after 'oral' doses of [^{3}H]pyridoxine HCl. Labelled pyridoxine was given through a fine polythene tube passed into the oesophagus of the unanaesthetized animal. The rats were starved for 24 hr before the test. After the oral doses had been given each rat was placed in a separate metabolism cage (Hanna & Allcock, 1961) and the urine passed during the ensuing 12 hr was collected. Specimens which were contaminated by faeces were discarded. The radioactivity in the urine samples was then measured according to the methods already described.

Six rats were given oral test doses of 0·05 mg of [^{3}H]pyridoxine, first before and then after previous 'saturation' with daily intraperitoneal injections of 10 mg unlabelled pyridoxine for the preceding 3 days. Since relatively little radioactivity was excreted when test doses were given in this way, these rats were then given similar oral test doses of [^{3}H]pyridoxine and at the same time parenteral injections of increasing amounts of non-radioactive pyridoxine were given intraperitoneally, a technique which was found greatly to increase the percentage of the dose excreted in the urine.

In order to determine the absorption of larger amounts of pyridoxine, six other rats were given increasing oral test doses of [^{3}H]pyridoxine (from 0·05 to 5·0 mg) together with a constant intraperitoneal injection of unlabelled pyridoxine, and the urinary excretion of radioactivity was measured.

Nature of urinary radioactivity. The amount of radioactivity in the urine was relatively low when test doses labelled with 1 μc of tritium were used. Four rats were therefore given 0·05 mg of pyridoxine labelled with 40 μc of tritium, in order to obtain highly radioactive urine samples. Two of these rats received this dose only; the other two animals were given a simultaneous injection of 10 mg of unlabelled pyridoxine to increase the urinary excretion of radioactivity. The urine was collected for 12 hr as before. Carrier pyridoxine HCl at a concentration of 5 mg/ml. and 4-pyridoxic acid at 1 mg/ml. were then added to the urine samples and 10 μl. volumes were subjected to electrophoresis in carbonate buffer at pH 11·1.

Rate and site of absorption of [^{3}H]pyridoxine. Two groups of twelve animals were used. Test doses of 0·05 mg [^{3}H]pyridoxine were given to the first group of animals; the second group received doses of 5·0 μg [^{3}H]pyridoxine. Three animals from each group were then killed by ether anaesthesia at each of the following times after the oral test doses: 7½, 15, 30 and 60 min. After death, the gastro-oesophagal junction, pylorus and terminal ileum were clamped and the stomach and entire small intestine were removed. The small intestine was divided into four segments of equal length as follows: (1) duodenum and upper jejunum; (2) lower jejunum; (3) upper ileum; (4) lower ileum. The contents of the stomach and each segment of the small intestine were then washed out with 10 ml. of normal saline and the amount of radioactivity in the washings was measured.

Effect of resection of the distal small intestine. Absorption tests were also carried out in the five animals in which between 50 and 70 % of the distal small intestine had been resected. These animals were given successive oral doses of between 0·05 and 5·0 mg of [^{3}H]pyridoxine together with an intraperitoneal injection of 10 mg of unlabelled pyridoxine. The urinary excretion of radioactivity was measured as in normal rats.

Absorption from the stomach was studied in three rats in which the gastro-oesophagal junction and pylorus had been ligated under ether anaesthesia, and in which 0·05 mg of [^{3}H]pyridoxine (contained in 0·5 ml. volume) was injected into the stomach. The animals were allowed to recover and were killed half an hour later. The stomachs were washed out with normal saline and the radioactivity in the washings was measured.

Comparison of absorption from jejunum, ileum and colon. Three groups of five rats were used. Under ether anaesthesia 0·05 mg of [^{3}H]pyridoxine contained in a volume of 0·25 ml. was injected from a graduated Mantoux syringe directly into the upper jejunum, upper ileum or caecum. At the same time 10 mg of unlabelled pyridoxine was given intraperitoneally, to ensure maximal excretion of radioactivity. The animals were then placed in separate metabolism cages and allowed to recover. Urinary radioactivity was measured as already described.

RESULTS

Electrophoresis of [^{3}H]pyridoxine HCl

[^{3}H]pyridoxine, to which carrier pyridoxine at a concentration of 5 mg /ml. had been added, was subjected to electrophoresis in acetate buffer at pH 5·1. The ultra-violet fluorescence due to pyridoxine and the distribution of radioactivity in the electrophoretic strip are shown in Fig. 1.

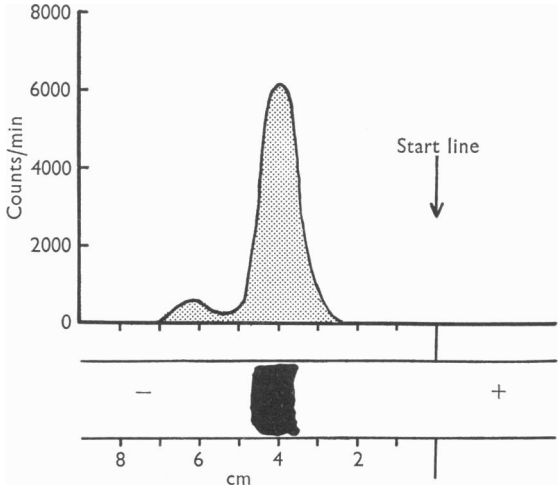

Fig. 1. Electrophoresis of [^{3}H]pyridoxine HCl in acetate buffer at pH 5·1. In Figs. 1, 2 and 4; above, distribution of radioactivity; below, ultra-violet fluorescence.

More than 50 % of the radioactivity corresponded to the position of pyridoxine HCl (Fig. 1). A small fraction (less than 5 %) was represented by a faster-moving component. This radioactivity did not correspond with any

known pyridoxine derivative and the nature of the impurity remains uncertain.

TABLE 1. Urinary radioactivity during 12 hr after oral doses of 0·05 mg [^{3}H]pyridoxine

Rat no.	Percentage of dose excreted	
	Before saturation	After saturation*
1	34·1	30·5
2	40·0	31·6
3	34·8	15·3
4	35·0	18·2
5	43·7	38·7
6	16·6	23·0
Mean ± S.D.	34·0 ± 8·9	26·2 ± 8·2

* Animals were 'saturated' by intraperitoneal injection of 10 mg pyridoxine HCl daily for 3 days.

Urinary excretion of radioactivity after oral doses

Effect of 'saturation' with pyridoxine HCl

Six rats (Nos. 1–6) were given test doses of 0·05 mg of [^{3}H]pyridoxine. They were then given the same test dose after previous 'saturation' with daily intraperitoneal injections of 10 mg unlabelled pyridoxine HCl for 3 days. The percentage of these doses excreted in the urine is given in Table 1. These animals excreted between 16·6 and 43·7 % (mean 34·0 %) of the first test dose, and there was no significant difference when the doses were given after the animals had been 'saturated' (Table 1).

Nature of urinary radioactivity. The results of electrophoresis of the urine collected after an oral dose of 0·05 mg of pyridoxine HCl labelled with 40 μc of tritium are shown in Fig. 2. Unlabelled pyridoxine HCl and 4-pyridoxic acid were added to the urine as markers. Approximately 60 % of the excreted radioactivity corresponded to the position of pyridoxine HCl. The remainder moved to a position between that of pyridoxine and 4-pyridoxic acid (Fig. 2).

Effect of simultaneous parenteral injection of increasing amounts of unlabelled pyridoxine HCl

The six rats (Nos. 1–6) were next given successive test doses of 0·05 mg of [^{3}H]pyridoxine and at the same time injections of unlabelled pyridoxine was given intraperitoneally, the parenteral injection being increased from 0·5 to 8·0 mg. The amounts of radioactivity excreted are illustrated in Fig. 3.

The amount of radioactivity excreted when the dose was given alone ranged from 18·2 to 38·3 % (Fig. 3). When a parenteral injection of non-labelled pyridoxine was given at the same time as the test dose was fed, larger amounts of radioactivity were excreted. The mean excretion after a parenteral dose of 0·5 mg was 54·8 % (range 31·2–70·2). When larger

parenteral injections were given the urinary excretion of radioactivity increased, the mean excretion reaching a maximum of 83·6% (range 80·0–94·0) when 8·0 mg was injected (Fig. 3).

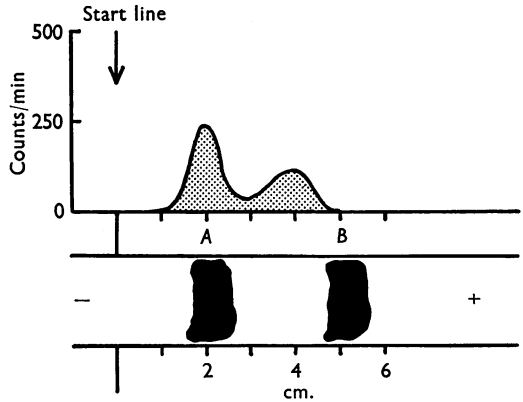

Fig. 2. Electrophoresis of urine after oral dose of [^{3}H]pyridoxine HCl given alone. Pyridoxine HCl (*A*) and 4-pyridoxic acid (*B*) have been added as markers. Carbonate buffer at pH 11·1.

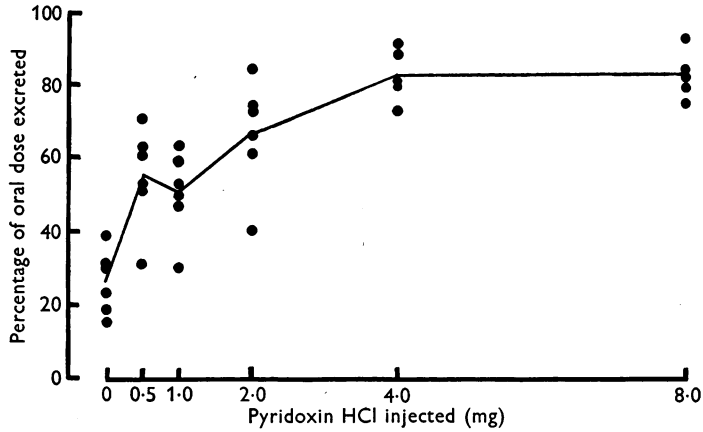

Fig. 3. Effect of simultaneous parenteral injections of non-radioactive pyridoxine HCl (from 0·5 to 8 mg) on the urinary excretion of radioactivity in 24 hr following an oral dose of 0·05 mg [^{3}H]pyridoxine HCl.

Nature of urinary radioactivity. Electrophoresis of the urine collected after an oral dose of 0·05 mg of pyridoxine HCl labelled with 40 μc of tritium, accompanied by a parenteral injection of 8 mg pyridoxine, was carried out as already described. The results are shown in Fig. 4. Most of the urinary radioactivity corresponded to pyridoxine HCl. There was also a small amount of the faster moving component already noted (Fig. 2).

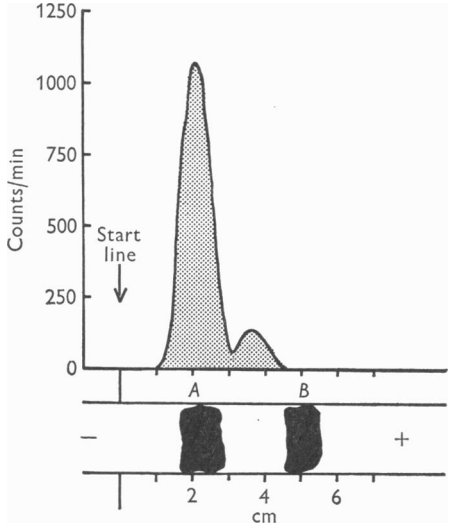

Fig. 4. Electrophoresis of urine after oral dose of [^{3}H]pyridoxine HCl given with simultaneous parenteral injection of 10 mg non-radioactive pyridoxine. Markers and buffer as in Fig. 2.

TABLE 2. Percentage of [^{3}H]pyridoxine excreted in urine by normal rats during 12 hr after oral doses of 0·05–5 mg*

	[^{3}H]pyridoxine fed (mg)				
Rat no.	0·05	0·1	0·5	1·0	5·0
7	86·0	83·9	73·0	79·0	63·5
8	59·8	72·2	53·0	73·5	56·0
9	74·0	72·3	52·1	76·2	78·5
10	60·0	77·0	53·5	59·0	73·5
11	89·0	80·2	56·8	62·7	74·5
12	—	73·0	—	—	63·5
Mean ± S.D.	73·7 ± 12·4	76·4 ± 4·4	57·7 ± 7·8	70·1 ± 7·8	68·2 ± 7·8

* 10 mg of unlabelled pyridoxine HCl was given intraperitoneally at the same time as the oral doses were fed.

Increasing oral doses of [^{3}H]pyridoxine HCl

Six other rats (Nos. 7–12) were given increasing oral test doses of [^{3}H]-pyridoxine (from 0·05 to 5·0 mg) together with an intraperitoneal injection of 10 mg of unlabelled pyridoxine to ensure maximal excretion of radioactivity. The percentages of these doses excreted in the urine are given in Table 2 and the actual amounts of radioactivity excreted, expressed as mg of [^{3}H]pyridoxine, are illustrated in Fig. 5. As Table 2 shows, there was little difference in the percentage of the dose excreted in the urine, irrespective of the size of the oral test dose. The mean excretion after a

dose of 0·05 mg was 73·7 % (range 59·8–89·0) and the percentage excretion was not significantly different after a 5·0 mg dose (range 56·0–78·5, mean 68·2 %). In terms of the actual amount of pyridoxine excreted, however, there was a progressive and linear increase in the excretion with increase in the amount of pyridoxine fed (Fig. 5).

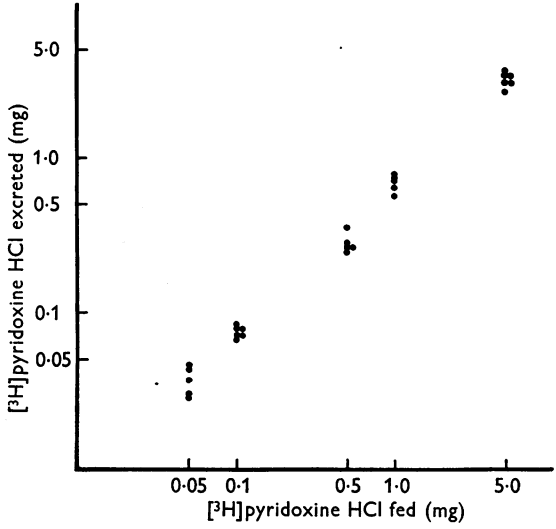

Fig. 5. Amounts of [³H]pyridoxine HCl excreted in the urine of normal rats during 24 hr after increasing oral doses of [³H]pyridoxine (from 0·05 to 5·0 mg). A 'flushing' injection of 10 mg was given simultaneously.

Relationship between rate and site of absorption

Rate of absorption

The absorption by the animals killed at intervals of 7½–60 min after being given either 0·05 or 5·0 mg of [³H]pyridoxine was calculated by assuming that the radioactive material not recovered had been absorbed. The percentage of the dose absorbed by each rat is illustrated in Fig. 6; the mean absorption at the varying time intervals after feeding is also shown. The initial rate of absorption of pyridoxine was remarkably rapid and both doses were absorbed at a similar rate (Fig. 6). By 15 min the mean absorption was 74·7 % after the 0·05 mg dose and 69·2 % after the dose of 5·0 mg. After this time absorption continued more slowly, so that by 60 min more than 90 % had been absorbed from either dose (Fig. 6).

Distribution of radioactivity in the gastro-intestinal tract

The mean percentage of the administered radioactivity found in the stomach and the four different segments of the small intestine of the three

rats killed at each time are shown in Fig. 7. The range of results in each group of animals is also shown.

At 7½ min after the doses were fed most radioactivity was present in the stomach (Fig. 7). The gastric radioactivity had fallen considerably by 15 and 30 min, and at 60 min after either dose less than 2 % of the administered pyridoxine remained.

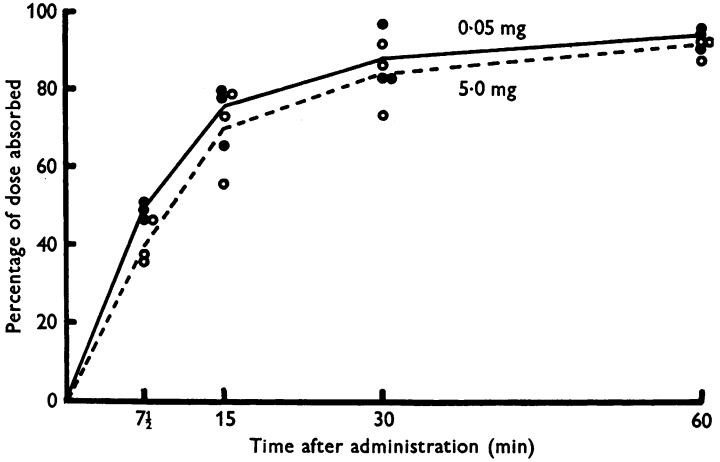

Fig. 6. Percentage absorption of [^{3}H]pyridoxine HCl by rats killed at varying intervals of time after receiving oral test doses of 0·05 (●) or 5·0 mg (○). Mean absorption at each time is indicated by the two lines.

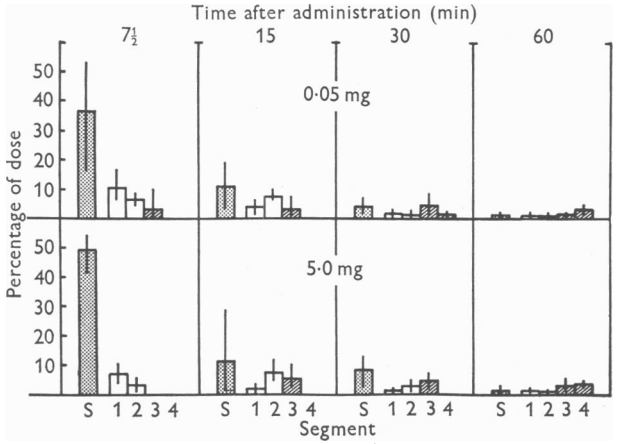

Fig. 7. The columns represent the mean amount of radioactivity in stomach ▦ and four equal segments of the small intestine of three rats killed at intervals of 7½–60 min after receiving 0·05 or 5·0 mg of [^{3}H]pyridoxine HCl orally. The intestinal segments were numbered as follows: (1) Duodenum and upper jejunum □; (2) lower jejunum □, (3) upper ileum. ▨; (4) lower ileum ▨ The range of results is indicated by the vertical line.

Despite the rapid gastric emptying, the amounts of either dose recovered from segments 1 and 2 were rarely as high as 10% (Fig. 7). The third segment contained virtually no radioactivity after either dose at 7½ min, except in a single animal. At 15 min the mean amount of radioactivity in this segment had risen to 3·1% after the 0·05 mg dose and to 5·9% after the larger dose. Less than 5% was present in segment 3 in any of the animals at 30 or 60 min.

Insignificant amounts of radioactivity were found in the fourth segment at 7½ or 15 min after either test dose. After half an hour a small percentage was recovered from this segment in two of the animals given the smaller dose, but there was no radioactivity in the fourth segment at this time following the 5·0 mg dose; by 60 min the mean radioactivity in segment 4 had risen to 3·2% after the 0·05 mg dose and to 3·3% after the larger dose (Fig. 7).

Effect of resection of the distal small intestine

The animals in which between 50 and 70% of the distal small intestine had been resected were given test doses of between 0·05 and 5·0 mg of [^{3}H]pyridoxine. They also received an intraperitoneal injection of 10 mg unlabelled pyridoxine to ensure maximal excretion of radioactivity. The percentage of the oral dose excreted in the urine is given in Table 3; the actual amount of labelled pyridoxine excreted is shown in Fig. 8. The amounts of [^{3}H]pyridoxine excreted in the urine by these animals (Fig. 8) were similar to those in the normal animals in which no resections had been carried out (Fig. 5).

TABLE 3. Percentage of [^{3}H]pyridoxine excreted in urine during 12 hr after oral doses of 0·05–5·0 mg* by rats subjected to resection of distal small intestine

Rat no.	[^{3}H]pyridoxine fed (mg)				
	0·05	0·1	0·5	1·0	5·0
37	77·1	78·0	62·6	60·8	75·0
38	66·0	77·0	71·5	79·0	78·0
39	50·5	84·1	87·5	—†	—†
40	67·0	80·5	85·1	67·7	84·0
41	—†	77·0	76·9	—†	—†
Mean	65·1	79·3	80·6	69·2	78·2

* 10 mg of unlabelled pyridoxine HCl was given intraperitoneally at the same time as the oral doses were fed. † Urine contaminated with faeces during experiment.

Comparison of absorption from stomach, jejunum, ileum and colon

Stomach. The percentages of the dose recovered from the stomachs of the three rats given 0·05 mg of labelled pyridoxine intragastrically, with the pylorus and oesophagus ligated, were 99, 101, and 98%, respectively, indicating that no significant absorption of pyridoxine occurs from the stomach.

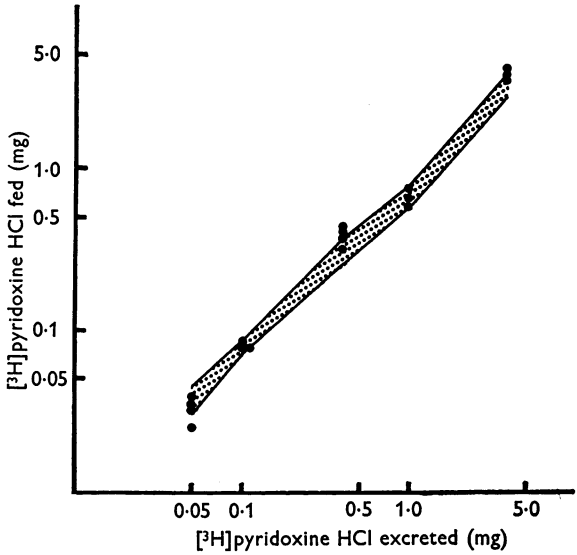

Fig. 8. Amount of [^{3}H]pyridoxine HCl excreted in the urine of rats subjected to resection of the distal small intestine during 24 hr after increasing oral doses of [^{3}H]pyridoxine HCl from 0·05 to 5·0 mg. A 'flushing' intraperitoneal injection of 10 mg was given simultaneously. The range of results in normal animals is indicated by the hatched area. Each point represents the amount of [^{3}H]pyridoxine excreted by a single resected animal.

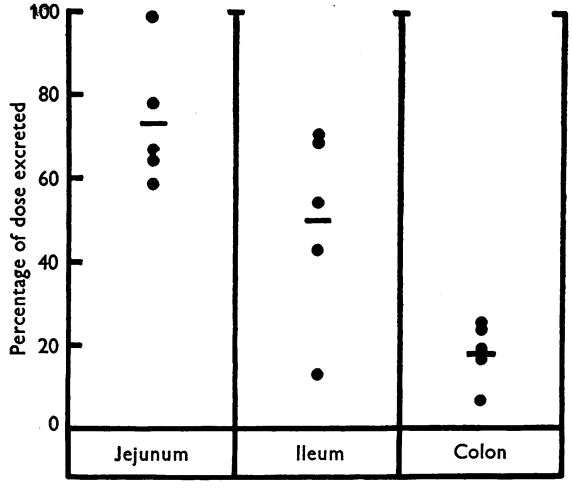

Fig. 9. Comparison of urinary excretion of radioactivity after 0·05 mg of [^{3}H]-pyridoxine was introduced directly into jejunum, ileum or colon.

Jejunum, ileum or colon. The amounts of radioactivity excreted in the urine by each group of five rats given 0·05 mg [^{3}H]pyridoxine directly into the jejunum, ileum or caecum are shown in Fig. 9; an intraperitoneal injection of 10 mg unlabelled pyridoxine was given at the same time to ensure maximal excretion of radioactivity. From 59 to 99 % (mean 71%) was excreted when the dose was given into the upper jejunum (Fig. 9). The amount excreted after injection into the ileum of the test dose ranged from 13 to 70 % (mean 50 %). Very much less radioactivity was excreted when the dose was given into the caecum (range 7–27 %, mean 18 %) (Fig. 9).

DISCUSSION

The results given in this paper illustrate that [^{3}H]pyridoxine HCl may be used for absorption studies. When the radioactive vitamin is given to rats orally, relatively little radioactivity is excreted in the urine (Table 1). However, if the oral test dose is accompanied by a large parenteral injection of non-radioactive pyridoxine HCl very much larger amounts of radioactivity are excreted (Fig. 3, Table 2). With this technique it is possible to study absorption by measuring the urinary excretion of radioactivity after giving an oral test dose of [^{3}H]pyridoxine HCl.

The results indicate that the intestinal tract of the rat has a remarkable capacity to absorb pyridoxine. When increasing doses of labelled pyridoxine (from 0·05 to 5·0 mg) are given, increasing amounts are excreted in the urine, indicating an increasing absorption (Fig. 5). The daily pyridoxine requirement of a 200 g rat has been estimated to be approximately 10 μg (Brown & Sturtevant, 1949). Yet the rat is clearly able to absorb as much as 500 times its daily requirement with ease (Fig. 5). This extraordinary capacity for pyridoxine absorption in the rat appears to be a function of the jejunum. As is shown by the results in Fig. 6, absorption of either a small or a large dose of pyridoxine occurs very rapidly and this absorption takes place in the jejunum regardless of the size of the dose, relatively little radioactivity ever reaching the ileum when either 0·05 or 5·0 mg of pyridoxine HCl was fed (Fig. 7). The results in the rats subjected to resection of the distal small intestine confirm the importance of the jejunum in the absorption of pyridoxine, for even when the largest doses were given to these animals, absorption was as complete as in normal rats (Fig. 8).

This response of the jejunum to increasing oral doses of pyridoxine suggests that absorption occurs by diffusion. For if absorption of a substance occurs through an active transport mechanism, it may be possible to saturate the absorptive capacity of this system. In the case of a substance normally absorbed proximally by such a mechanism, the site of absorption becomes progressively more distal with increase in the dietary

load. Such a response of the small intestine has been demonstrated for fat (Booth, Read & Jones, 1961; Booth, Aldis & Read, 1961), a substance which is generally considered to be absorbed by active mechanisms. The failure of even 5 mg of pyridoxine to saturate any more of the jejunum than the dose of 0·05 mg suggests that there may be no transport mechanism and that absorption may be occurring by diffusion. This conclusion is supported by unpublished observations of J. S. Stewart and C. C. Booth on everted sacs of rat intestine, which have failed to demonstrate any active transport system for pyridoxine absorption *in vitro*. Although the jejunum appears to be the major site of absorption, the ileum is also capable of absorbing this vitamin and some absorption may occur from the colon when pyridoxine is directly introduced into this organ. These observations are in keeping with a diffusion mechanism of absorption. Evidence suggests that other water-soluble vitamins of the B group may be absorbed in the same way (Morrison & Campbell, 1960; Spencer & Zamcheck, 1961; Turner & Hughes, 1962).

The labelled pyridoxine used in this study appears to be stable and was not broken down by absorption or by metabolism in the body, for most of the radioactivity excreted in the urine was shown to be due to labelled pyridoxine (Figs. 2 and 4). Furthermore, the great increase in urinary excretion when intraperitoneal injections of non-radioactive material are used to 'flush' radioactivity from the body suggests that the labelled pyridoxine behaves in the same way as the unlabelled vitamin.

The urine also contained small amounts of radioactivity which did not appear to correspond electrophoretically to any known derivative of pyridoxine but which was present in the original labelled material (Fig. 1). The nature of this radioactivity remains to be determined.

SUMMARY

1. The absorption of oral doses of tritium-labelled pyridoxine HCl (from 0·05 to 5·0 mg) was studied in the rat. The results indicate that pyridoxine is absorbed rapidly from the upper intestine regardless of the size of the dose given. Absorption may also occur from the ileum and to a small extent from the colon.

2. There is a linear relationship between oral dose and the amount absorbed in normal animals and in those in which the distal small intestine has been resected. These observations suggest the possibility that pyridoxine may be absorbed by diffusion.

3. The tritium-labelled material used in this study was more than 95 % pure. The label did not appear to become detached during absorption or passage through the body. Urinary excretion experiments with a simul-

taneous parenteral injection of unlabelled pyridoxine suggested that the radioactive vitamin behaved in a similar way to the non-radioactive pyridoxine.

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