INTESTINAL ABSORPTION OF VITAMIN B₆

I. PYRIDOXOL UPTAKE BY RAT INTESTINAL TISSUE¹

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(Received August 22, 1973)

The uptake of ³H-pyridoxol by everted intestinal rings of the rat was studied mainly at concentrations of 0.1–4 μM of the vitamin in the medium. Incubation of the tissue at 37°C for 60 min in Krebs-Ringer bicarbonate medium containing glucose and labelled pyridoxol resulted in intracellular accumulation of the labelled compounds. The total intracellular concentration of the compounds after the incubation was always higher than the extracellular concentration, and this accumulation was especially appreciable at relatively low external concentrations of the ³H-vitamin. The uptake under the same conditions was partially saturated with increasing concentration of ³H-pyridoxol in the medium, whereas the uptake during the initial 5 min of incubation increased in a linear manner with an increase of external concentration. The uptake was markedly depressed by lowering the temperature of incubation or addition of 4-deoxypyridoxol. The inhibition by 4-deoxypyridoxol affected the time course of uptake mainly in its later period, and also depressed both the linear and saturable components contained in the relation of uptake to the external concentration of the ³H-vitamin. However, a considerable uptake occurred even at the maximum inhibition with 4-deoxypyridoxol. Examination of the intracellular forms of the ³H-vitamin taken up revealed that the major components were three B₆ phosphates. This phosphorylating tendency was particularly extreme when the tissue was incubated under conditions allowing marked accumulation of the ³H-vitamin. Of these phosphate esters, ³H-pyridoxamine 5'-phosphate appeared most highly responsible for the accumulation. 4-Deoxypyridoxol remarkably diminished the ³H-vitamin B₆ phosphates with a consequent decrease of total intracellular ³H-vitamin B₆ compounds, although it permitted free ³H-pyridoxol increase. From these results it is concluded that pyridoxol enters the intracellular space by simple diffusion, is accumulated by metabolic conversion to the phosphate forms of

¹ This paper was presented at the 25th Meeting of the Vitamin Society of Japan.

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the vitamin, and the conversion is inhibited by 4-deoxypyridoxol.

The intestinal absorption of POL³ has been demonstrated to occur rapidly and completely in man, the dog, the rat (1-4), and the rabbit (5). A simple diffusion mechanism of absorption has been strongly suggested by the linear relationship found between oral dose and the amount absorbed in man and the rat (2, 3), and also by the similar results of experiments *in vitro* with everted sacs of rat intestine (2). The possibility of active participation of the intestine in POL absorption was suggested by the finding that a part of the vitamin taken up by isolated rabbit intestinal tissue was present in the form of PAL-P. However, the relation of POL uptake to the dose applied did not support the suggestion, but was rather consistent with the simple diffusion mechanism (5).

Recent studies on the intestinal absorption of thiamine have demonstrated that an active transport mechanism works when as low a level of thiamine as 1 μ M was used (6). In this respect, the previous studies on POL absorption were mostly made using relatively large amounts; at least about a daily requirement of experimental animals was applied at once. It seems, therefore, of interest to find out if simple diffusion is the exclusive mechanism of absorption even when the POL available is so limited that any active function of the intestine might be of physiological importance.

In order to obtain information on the absorption under the conditions described above we have studied the uptake of tritiated POL from very dilute solutions by the intestinal tissue of the rat. The use of small ring segments of the intestine has been chosen because this method makes it possible to divide the tissue randomly into groups and therefore avoid any variation of the uptake with individual animals and sites in the intestine. The present paper reports that the tissue accumulates POL mainly in its phosphate forms and thus attains an apparent uphill transport with respect to all B₆ compounds.

MATERIALS

Chemicals. ³H-POL was obtained from the Radiochemical Centre (England), and was diluted with unlabelled POL to achieve a specific radioactivity of 52 or 330 mCi/mmole. This preparation has been reported to lose about 10% of the tritium when the vitamin is metabolized to pyridoxic acid (7). Therefore, it was assumed that about 5% of the radioactivity was lost by the metabolic conversion to PAL, PAM, and their phosphate forms, and based on this assumption corrections were made for the specific radioactivity of these ³H-POL derivatives when specifically noted. POL-P was a gift from Drs. S. Fukui and F. Masugi, Kyoto

³ Abbreviations used are: POL, pyridoxol; PAL, pyridoxal; PAM, pyridoxamine; PiA, 4-pyridoxic acid; DOPOL, 4-deoxypyridoxol; POL-P, pyridoxol 5'-phosphate; PAL-P, pyridoxal 5'-phosphate; PAM-P, pyridoxamine 5'-phosphate.

University. PiA was purchased from the Sigma Chemicals Co. (U.S.A.), and other vitamin B₆ compounds and DOPOL were obtained from Nakarai Chemicals Co. (Kyoto). (¹⁴C-Hydroxymethyl)-inulin (9.8 mCi/mmol) was obtained from the Radiochemical Centre (England). All the other chemicals used were purchased from commercial sources.

Animals. Male rats, Wistar strain, weighing between 150 and 250 g were used.

METHODS

Preparation of everted intestinal rings. The method is essentially similar to that employed by KOMAI and SHINDO (6). Animals that had been starved for 22 hr were decapitated after ether anesthesia, and the small intestine was removed, everted, and cut into rings of 3–5 mm length, which were kept in 0.9% NaCl at 37°C before use.

Uptake experiments. About 15 intestinal rings (about 0.3 g wet weight) were placed in a 100-ml Erlenmeyer flask with 15 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 0.4% glucose, and the mixture was incubated at a temperature to be described on a shaking waterbath under bubbling of a mixture of O₂ and CO₂ (95:5, v/v), through the medium. After 10 min, an appropriate concentration of ³H-POL (52 mCi/mmol) in a small volume of water (at most 0.6 ml) was added, the flask was stoppered, and the incubation was continued for the desired period. At the end of incubation the tissue was separated from the medium with a close-meshed sieve, rapidly rinsed three times with 75 ml of ice-cold 0.9% NaCl, blotted with filter paper, weighed and homogenized in 3 ml of 10% perchloric acid with a Potter-Elvehjem homogenizer. The medium was kept for radioactivity measurement. The homogenate was centrifuged at 40,000×g for 15 min, the precipitate reextracted with 3 ml of 10% perchloric acid, and the combined supernatants were neutralized with 4 N KOH. After standing at 0°C for 15 min, 1 ml of the supernatant was taken out for radioactivity measurement, the precipitated potassium perchlorate was removed by filtering through a sintered glass filter under suction to estimate the total volume of the extract.

In experiments to test the effects of inhibitors, DOPOL was added to the incubation mixture at the same time as ³H-POL, and the other inhibitors were added 9 min before that.

Determination of different ³H-vitamin B₆ compounds in the tissue. To obtain intestinal rings containing ³H-B₆ compounds the same procedure as described above was employed up to the step of blotting the tissue except that the ³H-POL used had a higher specific radioactivity (330 mCi/mmol) and rings prepared from a whole small intestine (about 3 g wet weight) were incubated in 120 ml of the medium. The blotting was performed in a cold room (4°C) to avoid further

metabolic conversion of B₆ compounds. The tissue was then placed in a weighing bottle containing a known weight of cold 5% trichloroacetic acid, and the total weight was measured at room temperature. Following homogenization in 24 ml of 5% trichloroacetic acid, the homogenate was centrifuged and the precipitate reextracted with the same volume of the acid solution. The combined supernatants were washed 5 times with ether using 100 ml each time. The pH of the resultant solution (about pH 3.6) was adjusted to 4.0 with 0.01 N NaOH, then the solution was lyophilized in the dark, and the residue was dissolved in 2 ml of water.

Paper chromatography on Whatman No. 3 MM filter paper was employed for the separation of ³H-B₆ compounds in the tissue extract. Usually 0.1 ml of the tissue extract was applied by streaking in a range of 11 cm width, and authentic samples to be cochromatographed were spotted at appropriate positions on the streak of the extract. Chromatography was run in the dark for about 16 hr, using Solvent A, *t*-butanol-acetone-diethylamine-water (40: 35: 5: 20, v/v) or Solvent B, *n*-butanol-formic acid-water (70: 15: 15, v/v).

After recording the position of the individual authentic B₆ compounds, revealed by exposure to ultraviolet light, the paper was cut into strips 1 cm wide, and the radioactive compounds were extracted with water for counting. The recovery of the radioactivity in the water extract was usually about 90% of that applied.

Calculation of intracellular concentrations of ³H-compounds. Intracellular concentration of ³H-B₆ compounds was calculated according to the following equation.

$$\text{Intracellular concentration } (\mu\text{M}) = Ct - CmK/W - K$$

where

Ct = total ³H-compounds in tissue (nmoles/g wet weight)

Cm = final concentration of ³H-compounds in medium (nmoles/ml)

K = extracellular fluid space in tissue determined with (¹⁴C-hydroxymethyl)-inulin (ml/g wet weight)

W = tissue water (ml/g wet weight)

The tissue water determined by drying wet tissue at 70°C for 24 hr was 0.818 ± 0.010 ml/g wet weight (mean $\pm$ standard deviation for 4 samples). For determination of extracellular fluid space, the same procedure as used for the determination of ³H-POL uptake was followed except that (¹⁴C-hydroxymethyl)-inulin was used in place of ³H-POL, and the space was calculated from the final radioactivities in the medium and tissue. Values obtained by incubation at 37°C for 5, 10, 30, and 60 min were 0.126 ± 0.014 , 0.185 ± 0.018 , 0.246 ± 0.035 , and 0.268 ± 0.002 ml/g wet weight (means $\pm$ standard deviation for at least 2 samples), respectively. In the case of incubation at 0°C, determination of the extracellular fluid space was always carried out in parallel with the uptake experiments of ³H-POL, since small fluctuation of the space seriously affected the estimation of

intracellular $^3\text{H-B}_6$ compounds owing to the small size of the $^3\text{H-POL}$ uptake. Typical values of the space obtained by incubation at 0°C for 5, 10, 30, and 60 min were 0.0795, 0.124, 0.227, and 0.289 ml/g wet weight, respectively.

In the experiments to determine individual intracellular $^3\text{H-B}_6$ compounds, the distribution of the compounds in the medium after incubation was first examined by paper chromatography with the same solvent as used for extracts, and the intracellular concentration was calculated from the content in the tissue based on the extracellular concentration of individual compounds thus obtained.

Radioactivity measurements. To 1 ml of each sample 10 ml of BRAY's solution (8) was added, and the radioactivity was determined in a liquid scintillation spectrometer (Packard model, 3375, Tri-carb). The efficiency for tritium in this system was in a range of 16–20%. Correction for quenching was made by the external standardization method.

RESULTS

General properties of uptake system

Figure 1 shows $^3\text{H-POL}$ uptake into the intracellular fluid space of the tissue and $^3\text{H-POL}$ concentration in the medium as functions of time when the tissue was incubated with $2\ \mu\text{M}$ $^3\text{H-POL}$ at 37°C . There was a linear increase in the

Fig. 1. Time course of $^3\text{H-POL}$ uptake by everted intestinal rings of the rat and $^3\text{H-POL}$ concentration in the medium. The tissue was incubated with $2\ \mu\text{M}$ $^3\text{H-POL}$ at 37°C for various periods as indicated. Other details of the determination of uptake are described in METHODS. The results are expressed as $^3\text{H-POL}$ concentration, (1) in the intracellular fluid space of the tissue, (2) in the medium. Each point represents the mean of two determinations.

uptake during the first 10 min of incubation, after which it increased more slowly but continued to increase until 60 min. The intracellular to extracellular concentration ratio of total $^3\text{H-B}_6$ compounds was found to be unity at about 30 min and reached about 1.6 at 60 min. This fact may be emphasized, since this is not expected to occur if simple diffusion is the only mechanism functioning in the uptake. The shape of the uptake curve as a whole suggests the possibility that the uptake involves at least two processes rather than only one. This possibility was investigated by examining the uptake during the first 5 min and 60 min of incubation at various external $^3\text{H-POL}$ concentrations. The former incubation period was chosen to observe the uptake within its linearly increasing phase, and the latter to observe the maximal uptake under the experimental conditions. The two curves in Fig. 2, which illustrate the relation of uptake during the two incubation periods to the final external $^3\text{H-POL}$ concentration, are obviously different; curve 1 shows a straight line, and the curve 2 looks like a hyperbola whose asymptote has a certain slope. Thus, the uptake during 5 min of incubation increased linearly with increasing external concentration, as shown by curve 1. On the

Fig. 2. Effects of incubation time on the relation of $^3\text{H-POL}$ uptake to its concentration in the medium. The tissue was incubated with $0.1\text{--}4\ \mu\text{M}$ $^3\text{H-POL}$ at 37°C for 5 or 60 min. Other details of the determination of uptake are described in METHODS. The uptake at (1) 5 min, and (2) 60 min, is plotted against final $^3\text{H-POL}$ concentration in the medium. Each point represents mean $\pm$ standard deviation of 4–14 determinations.

other hand, curve 2 shows that the uptake at 60 min rapidly increased with increasing external ^3H -POL concentration up to about $0.3\ \mu\text{M}$, and that at larger concentrations it increased more slowly and made an almost linear slope. The ratio of intracellular to extracellular concentration of total ^3H -B₆ compounds was correspondingly high at low external concentrations, *e.g.*, the ratio was about 3 at $0.2\ \mu\text{M}$, and decreased with increasing external concentration, but was always higher than unity in the range of concentrations examined.

The uptake during 5 min incubation was further examined at much higher concentrations of ^3H -POL in the medium. The results given in Fig. 3 indicate that the uptake increased linearly with increasing external concentration up to at least $100\ \mu\text{M}$, and above that concentration, the uptake increased more slowly but did not reach a plateau even at about $2,000\ \mu\text{M}$.

Fig. 3. Relation of ^3H -POL uptake during 5 min of incubation to the external ^3H -POL concentration ranging up to about $2,000\ \mu\text{M}$. Experimental details are as in Fig. 2 except the higher concentrations of ^3H -POL ($2\text{--}2,000\ \mu\text{M}$) used. Each point shown represents a value obtained from a single determination, and the results obtained at ^3H -POL concentrations below $50\ \mu\text{M}$ are not shown.

Curves similar in shape to curve 2, Fig. 2, have been shown with respect to the relation between the initial rate and the external concentration in thymidine uptake by Novikoff rat hepatoma cells (9) and in tryptophan uptake by slices of rat cerebral cortex (10). They have been interpreted to reflect two processes, carrier-mediated transport and simple diffusion. The same interpretation, however, cannot be applied to curve 2 in Fig. 2, since it is not concerned with the initial rate of uptake, but with the accumulation during the prolonged incubation period. Even so, it seems pertinent at least to conclude that the curve consists

of plural components reflecting plural processes. Furthermore, it may be most simply postulated that the processes involved in the uptake are dual: one is saturable with ^3H -POL, as reflected by the hyperbolic character of the curve, and the other is nonsaturable in the range of ^3H -POL concentrations in Fig. 2, as reflected by the linear part of curve 2 and also by curve 1. The saturable process would have an obviously important role in the concentrative accumulation of ^3H -B₆ compounds, operating throughout the prolonged incubation period of 60 min, but would have no decisive role in the uptake during the initial 5 min of incubation as seen from the absence of hyperbolic character in curve 1. The nonsaturable process postulated might be saturated at much higher ^3H -POL concentrations, as suggested by the results in Fig. 3, or the results may mean that the process is in reality composed of more processes than the hypothesis described above.

Effects of temperature and inhibitors

A typical time course for uptake of ^3H -POL at 0°C is shown by curve 2 in Fig. 4. The uptake was very low throughout the incubation period, demonstrating

Fig. 4. Effects of DOPOL or low temperature on the time course of ^3H -POL uptake. The tissue was incubated with $2\ \mu\text{M}$ ^3H -POL, (1) in the presence of $200\ \mu\text{M}$ DOPOL at 37°C, and (2) at 0°C. Other details are as in Fig. 1. Each point in curve 1 represents the mean of two determinations, and those in curve 2 represent the mean of determinations.

a high sensitivity of the uptake system to temperature. No further analysis was made since the data badly scattered due to the very low ratio of the intracellular to extracellular contents of total ^3H -B₆ compounds.

A time course study was also made in the presence of DOPOL, which is known to be an effective inhibitor of B₆ kinase (11) and was also shown to inhibit POL uptake by *E. coli* (12), ascites-sarcoma cells (13), and human erythrocytes (14). The results of incubation at 37°C with $2\ \mu\text{M}$ ^3H -POL and $200\ \mu\text{M}$ DOPOL are

illustrated by curve 1 in Fig. 4. It is qualitatively indicated that the inhibitory effect of DOPOL is low in the early period of uptake and quite remarkable in the later period. In order to estimate quantitatively the effect of DOPOL, the inhibited uptake was compared with controls in a set of experiments. As shown in Table 1, the uptake even at 5 min was significantly inhibited, although the inhibition was much less than that of 60-min uptake. The results confirm that DOPOL affects mainly the later phase of the uptake, but the question remains whether it affects the real initial phase of uptake. Some commonly used inhibitors were also tested for their effects on the 60-min uptake at 37°C. No appreciable inhibition was observed with 2,4-dinitrophenol and ouabain even at concentrations

Table 1. Effect of inhibitors on ³H-POL uptake by everted intestinal rings of the rat.^a

Inhibitor	Incubation time (min)	³ H-POL uptake (μM × 1,000)		Inhibition (%)
		Expt. 1	Expt. 2	
None	5	496 ^b		—
	60	2,580	2,520	—
DOPOL, 0.2 mM	5	372		24.9
	60	1,200		53.5
2,4-Dinitrophenol, 1 mM	60		2,680	—6.2
Ouabain, 0.5 mM	60		2,350	6.7
Iodoacetic acid, 1 mM	60		1,600	36.5

^a The tissue was incubated with 2 μM ³H-POL at 37°C for the indicated times. Other experimental details for the determination of uptake are described in METHODS.

^b Each value represents the mean of duplicate determinations.

Fig. 5. Effects of DOPOL on the relation of ³H-POL uptake to its concentration in the medium. The tissue was incubated at 37°C for 60 min with various concentrations of a mixture of ³H-POL and DOPOL (1: 100, molar ratio). Other details are as in Fig. 2. Each point shown represents a value obtained from a single determination.

of 1 mM and 0.5 mM, respectively. Only iodoacetic acid considerably inhibited the uptake at 1 mM concentration.

To obtain further information on the manner of inhibition by DOPOL, its effects on the relation between ^3H -POL uptake and the external ^3H -POL concentration were examined. The uptake during 60 min of incubation at 37°C was determined at various ^3H -POL concentrations in the presence of constant 100-fold concentrations of DOPOL to ^3H -POL concentration. The curve shown in Fig. 5 is almost a straight line and has little hyperbolic character compared with curve

Fig. 6. Effects of various concentrations of DOPOL on ^3H -POL uptake. The tissue was incubated at 37°C for 60 min with $0.3 \mu\text{M}$ ^3H -POL in the presence of 0–300 μM DOPOL. Other details are as described in METHODS. (A) ^3H -POL uptake is plotted against DOPOL concentration, (B) the reciprocal of inhibition rate based on the uptake without DOPOL is plotted against the reciprocal of DOPOL concentration. Each point represents the mean of two determinations.

2 in Fig. 2, and the slope of the linear part of the former is also smaller than that of the latter. The inhibition rate estimated, taking the latter curve as control, was relatively high at low external concentrations, *e.g.*, 67% at 0.2 μ M, and low at high concentrations, *e.g.*, 43% at 2 μ M. This difference in the inhibition rate dependent on the external concentration can be related to the lack of hyperbolic character in the "inhibited" curve. The fact that the addition of DOPOL depressed both the saturable and nonsaturable processes postulated above suggests that DOPOL inhibits both processes or one preceding the other. This also gives rise to the question if all the processes involved in the uptake are subject to inhibition of DOPOL.

To investigate this point the effects of various concentrations of DOPOL on ³H-POL uptake at 60-min incubation at 37°C was examined. The relation of the uptake to the concentration of DOPOL gave a typical hyperbolic curve (Fig. 6A) when 3, 30, or 300 μ M DOPOL was added to 0.3 μ M ³H-POL. The maximum inhibition was estimated to be 86% from the intercept on the ordinate in Fig. 6B, where the reciprocal of the inhibition rate was plotted against the reciprocal of DOPOL concentration. The results indicate that the tissue would take up 0.129 μ M of ³H-POL, which is 43% of the initial external ³H-POL concentration, even at an infinitive concentration of DOPOL. It is, therefore, assumed that the uptake involves some process that is independent of DOPOL.

This assumption, as well as the relatively low inhibitory effect of DOPOL on the uptake at 5 min, suggests the possibility that the real initial process of uptake may not be affected by the inhibitor. On the other hand, the remarkable effect of DOPOL depressing the uptake in the later period of its time course suggests that some important process for the accumulation of ³H-B₆ compounds follows the initial one and this process is sensitive to DOPOL. The close relation of this process to the saturable process postulated above seems evident from the effect of DOPOL of abolishing the hyperbolic character in the relation between the uptake and the external concentration of ³H-POL.

Another fact to be noted is the finding that the nonsaturable process postulated above is still inhibited by DOPOL, in contrast to the above assumption on the existence of a DOPOL-independent process. Therefore, the "nonsaturable process" must be considered to be, in fact, composed of plural processes, at least one of which is sensitive and the other, insensitive to DOPOL.

The present results considered together suggest the possibility that DOPOL inhibits the intracellular metabolism of ³H-POL but not the entrance of ³H-POL into the intracellular space.

Forms of intracellular ³H-vitamin B₆ compounds

In order to obtain information on the metabolism of the ³H-POL taken up, the distribution of radioactive B₆ compounds was examined after incubation of the tissue with ³H-POL under various conditions. Extraction and separation of

Fig. 7. Paper chromatography in Solvent A of extract from the tissue incubated with $3 \mu\text{M}$ ^3H -POL for 60 min at 37°C . Experimental details are described in METHODS. The top part shows the distribution of ^3H -compounds on the chromatogram, and the bottom part shows the position of individual unlabeled B_6 compounds cochromatographed with the extract. Radioactivity found in the regions (A) and (B) was taken as that of ^3H - B_6 phosphates and POL, respectively.

the compounds were carried out as described in METHODS above. Figure 7 shows a typical example of the distribution of the compounds on a paper chromatogram developed with Solvent A, together with the position of individual unlabeled B_6 compounds cochromatographed. Results of paper chromatography in the same solvent of all the other extracts examined were similar in that ^3H - B_6 phosphates were the major components, and the others except (^3H -POL) were scanty. The fact that almost all of the three B_6 phosphates remained at the origin of the chromatogram may be due to salts and other contaminants contained in the

extracts, since authentic B₆ phosphates alone migrated more significantly. However, this abnormality is not likely to have caused any serious errors because the other B₆ compounds fairly migrated, and the amount of total ³H-B₆ phosphates thus estimated was in good agreement with the total of the individual amounts of those separated with another solvent, as shown in Fig. 8. The intracellular concentrations of ³H-POL, total B₆ phosphates, and total ³H-B₆ compounds were calculated as described in METHODS above.

Fig. 8. Paper chromatography in Solvent B of extract from the tissue incubated with $0.3 \mu\text{M}$ ³H-POL for 60 min at 37°C. Experimental details are described in METHODS. The top part shows the distribution of ³H-compounds on the chromatogram, and the bottom part shows the position of individual unlabeled B₆ compounds cochromatographed with the extract. Radioactivity found in the regions (A), (B), and (C) was taken as that of PAM-P, POL-P, and PAL-P, respectively.

The results summarized in Table 2 show that the intracellular was lower than the extracellular ³H-POL concentration under all conditions examined. This indicates that no transport of ³H-POL occurred against a concentration gradient and excludes the possibility of an active transport mechanism. The table also shows that ³H-B₆ compounds were mainly present in phosphate forms except in

Table 2. Intracellular ^3H -vitamin B_6 compounds extracted from intestinal rings incubated with ^3H -POL under various conditions.^a

^3H -POL in medium ($\mu\text{M} \times 1,000$)		Addition	Incubation time (min)	Intracellular ^3H - B_6 compounds ($\mu\text{M} \times 1,000$)		
Initial	Final			POL	B_6 phosphates	Total
300	278		5	3.3	72.0	85.4
300	202		60	3.2	618	709
300	265	DOPOL, $20\mu\text{M}$	60	96.0	129	274
300	302	DOPOL, $300\mu\text{M}$	60	102	20.0	144
3,000	2,560		5	178	337	597
3,000	2,570		60	754	2,210	3,610

^a The tissue was incubated at 37°C with the indicated concentrations of ^3H -POL for the indicated times. B_6 compounds were separated with paper chromatography in Solvent A as shown in Fig. 7. Other details are described in METHODS.

cases where DOPOL was added, and in particular, this tendency was extreme at the initial ^3H -POL concentration of $0.3\mu\text{M}$, where relatively high accumulation of ^3H - B_6 compounds occurred during 60 min of incubation. This fact strongly suggests that the accumulation is due to metabolic conversion of ^3H -POL to B_6 phosphates. The suggestion is further supported by the effects of DOPOL also shown in Table 2; the depression of the total amount of ^3H - B_6 compounds despite the increase in the amount of intracellular ^3H -POL is obviously due to remarkable inhibition of metabolic conversion. It is also noted that the intracellular concentration of ^3H -POL was extremely low and almost constant independent of the incubation time at $0.3\mu\text{M}$ of the initial external concentration, whereas the concentration was significant and increased with time at $3\mu\text{M}$. These facts indicate that the rate of metabolic conversion is exceedingly higher than the rate of entrance into the intracellular space at $0.3\mu\text{M}$, whereas this is not the case at $3\mu\text{M}$. This explanation is further supported by the fact that the inhibition of the metabolic conversion with DOPOL resulted in the remarkable increase of intracellular ^3H -POL. It is, therefore, concluded that the metabolic conversion and entrance are completely separated, and this conclusion excludes the possibility of direct coupling of these two steps.

The distribution of the three phosphate forms of ^3H -vitamin B_6 was further examined by paper chromatography with Solvent B. This solvent system could separate not only the three B_6 phosphates from one another, but also all of them from POL, PAL, and PiA as shown in Fig. 8. Although PAM was not separated from B_6 phosphates, it was ignored since its presence was constantly insignificant as shown by paper chromatography with Solvent A. The results summarized in Table 3 show that all three ^3H - B_6 phosphates were present in significant amounts under all the conditions examined. The order of the amounts of individual ^3H - B_6 phosphates was

Table 3. Distribution of intracellular ³H-vitamin B₆ phosphates formed from ³H-POL taken up under various conditions.^a

Initial concentration of ³ H-POL in medium ($\mu\text{M} \times 1,000$)	Incubation time (min)	Intracellular ³ H-B ₆ phosphates ($\mu\text{M} \times 1,000$)		
		POL-P	PAL-P	PAM-P
300	5	13.3	45.6 ^b	16.1 ^b
300	60	155	254	234
3,000	5	94.0	175	81.3
3,000	60	800	1,030	454

^a Incubation conditions are same as in Table 2. B₆ phosphates were separated with paper chromatography in Solvent B as shown in Fig. 8. Other details are described in METHODS.

^b Concentrations of PAL-P and PAM-P were calculated based on the assumption that their specific radioactivity was 95% of that of ³H-POL (see the MATERIALS) in test.

at 0.3 μM of external ³H-POL concentration, and was

$$\text{PAL-P} > \text{POL-P} > \text{PAM-P}$$

at 3 μM . It may be further pointed out that: (a) ³H-PAM-P was 36% of the total ³H-B₆ phosphates when the tissue was incubated with 0.3 μM ³H-POL for 60 min, the conditions allowing a relatively high accumulation of ³H-B₆ compounds, whereas ³H-PAM-P was about 20% (20–23%) in all the other three cases shown in Table 3. (b) With the incubation time of 60 min, quantitative change of ³H-PAM-P was the poorest of the three in responding to the change of external ³H-POL concentration; it increased less than twice against the 10-fold increase of the external ³H-POL. The results therefore suggest that the intracellular formation of ³H-PAM-P is most closely related to the concentrative accumulation of ³H-B₆ compounds at low external ³H-POL concentrations, and also to the saturating behavior of the accumulation at 60 min with ³H-POL in the medium.

However, all the results on the distribution of ³H-B₆ compounds taken collectively indicate that curve 2 in Fig. 2 consists of many components, and, therefore, analysis of the nature of the curve requires more investigation despite its apparent simplicity.

DISCUSSION

The present results have established that an apparent concentrative accumulation of B₆ compounds occurs when the intestinal tissue of the rat is exposed to very dilute solutions of POL, and this is through metabolic conversion of POL mainly to B₆ phosphates as demonstrated in RESULTS above. This fact is of physiological importance whatever the transport mechanism is. Living animals would be able to extract POL to the intestine from foods containing a small amount of the vitamin and at least retain it in the tissue. The proposal of

active function of the intestine based on the formation of PAL-P in the tissue (5) has thus been substantially supported and clarified. An interesting question still remains: Are formed B₆ phosphates transferred across the intestinal wall, and therefore, is the metabolic conversion a part of the overall mechanism of absorption.

The mechanism of POL uptake has been shown to consist of two separate steps. The first is entrance into the tissue, and the second is the metabolic conversion of POL in the intracellular space mainly to phosphate forms of vitamin B₆. The significant intracellular presence of all three B₆ phosphates indicates that the second step is actually composed of many processes, which are probably arranged to make the following widely accepted pathway (15-18):

The essential role of this step in the accumulation of B₆ compounds has been demonstrated by the predominance of the phosphate forms in the intracellular ³H-B₆ compounds and the depression of the accumulation caused by the inhibition of ³H-POL metabolism with DOPOL. DOPOL probably inhibits the phosphorylation of POL, the first process in this step, as proved for B₆ kinase from many different sources (11). The results have also suggested that the formation of PAM-P may be most important for the accumulation at low concentrations of POL in the medium.

As for the first step, experimental results have not produced very conclusive evidence on the mechanism of the entrance process, but none of them have really disproved the simple diffusion mechanism previously proposed (2, 3). The active transport mechanism is excluded since the intracellular concentration of ³H-POL was constantly smaller than that of external ³H-POL. The facilitated diffusion mechanism of the entrance process appears to be supported by the following facts: (a) ³H-POL uptake during the first 5 min of incubation, which was within the range of linear increase with time, was significantly depressed by the addition of DOPOL; (b) the increase in the rate of uptake at 5 min with the increasing external ³H-POL concentration tended to be reduced at high concentrations above 100 μM. However, it is possible to explain this effect of DOPOL as the consequence of inhibition of the metabolism but not of the entrance process, since it can be considered that the linear increase of the uptake for the first 5 min (Fig. 1) was possible only because of the rapid decrease of intracellular ³H-POL by the metabolic conversion. Without the metabolic conversion, the uptake at 5 min would have been lower than that observed, since the serious increase of intracellular ³H-POL would have affected its concentration gradient between the intra- and extracellular solutions. This alternative explanation, based on the simple diffusion mechanism, is substantially supported by the fact that (a) ³H-POL taken up in 5 min was mainly in the ³H-B₆ phosphate forms in the intracellular space, (b) intracellular ³H-POL remarkably increased by the inhibi-

tion of the metabolism with DOPOL (Table 2). The analogous effect of the increase in the external ³H-POL concentration can be similarly explained as the consequence of saturation of the metabolic conversion. The simple diffusion mechanism is further supported by the demonstration that a considerable amount of ³H-POL can be taken up even under the maximal inhibition by DOPOL.

Curve 2 in Fig. 4 has suggested that the entrance of ³H-POL as well as its metabolic conversion is depressed by lowering the temperature. Such a result has often been regarded as evidence against the simple diffusion mechanism. This argument, however, cannot always be applied since LIEB and STEIN recently gave many examples of simple diffusion across biological membranes that showed remarkable temperature-sensitivity (19). Therefore, it may be concluded that POL enters the intracellular fluid space by simple diffusion, except in the unlikely case that two distinct mechanisms are involved in the uptake.

REFERENCES

- 1) SCUDI, J. V., UNNA, K., and ANTROPOL, W., *J. Biol. Chem.*, **135**, 371 (1940).
- 2) BRAIN, M. C., STEWART, J. S., and BOOTH, C. C., *Strahlentherapie, Sonderbeaende*, **5**, 475 (1962).
- 3) BOOTH, C. C. and BRAIN, M. C., *J. Physiol.*, **164**, 282 (1962).
- 4) WOLF, V. H., *Z. Vitaminforsch.*, **28**, 281 (1958).
- 5) YAMADA, Y., *Vitamins* (in Japanese), **17**, 438 (1959).
- 6) KOMAI, T. and SHINDO, H., *J. Vitaminol.*, **18**, 55 (1972).
- 7) CONTRACTOR, S. F. and SHANE, B., *Biochem. Pharmacol.*, **19**, 1669 (1970).
- 8) BRAY, G. A., *Anal. Biochem.*, **1**, 279 (1960).
- 9) PLAGEMANN, P. G. W. and ERBE, J., *J. Cell Biol.*, **55**, 161 (1972).
- 10) KIELY, M. and SOURKES, T. L., *J. Neurochem.*, **19**, 2863 (1972).
- 11) MCCORMICK, D. B. and SNELL, E. E., *J. Biol. Chem.*, **236**, 2085 (1961).
- 12) OYA, N., *Vitamins* (in Japanese), **41**, 222 (1970).
- 13) TSUJI, M., NISHIWAKI, J., and YAMADA, K., *Vitamins* (in Japanese), **42**, 222 (1970).
- 14) YAMADA, K. and TSUJI, M., *J. Vitaminol.*, **16**, 237 (1970).
- 15) SAKAMOTO, Y., *Vitamins* (in Japanese), **24**, 223 (1961).
- 16) JOHANSSON, S., LINDSTEDT, S., and TISELIUS, H.-G., *Biochemistry*, **7**, 2327 (1968).
- 17) CONTRACTOR, S. F. and SHANE, B., *Biochim. Biophys. Acta*, **230**, 127 (1971).
- 18) TISELINS, H.-G., *J. Neurochem.*, **20**, 937 (1973).
- 19) LIEB, W. R. and STEIN, W. D., in F. Bronner and A. Kleinzeller (Editors), *Current Topics in Membranes and Transport*, Academic Press, New York and London, Vol. 2, p. 1 (1971).