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N^6-(Δ^2-ISOPENTENYL)ADENOSINE METABOLISM IN MAN

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Abstract—After intravenous administration of N^6-(Δ^2-isopentenyl)adenosine-8-^{14}C (IPA-8-^{14}C) to human subjects, several metabolites were isolated along with a small quantity of unchanged IPA. The ultraviolet spectra and paper chromatography of purified metabolites led to the identification of 6-*N*-(3-methyl-3-hydroxybutylamino)purine, hypoxanthine, adenine and some N^6-alkylated adenines and *N*-alkylated xanthines. A large quantity of the radioactivity (50 per cent) from IPA-8-^{14}C was excreted in the form of non-ultraviolet absorbing compounds, suggesting saturation or cleavage or both of the purine portion of IPA. Orally administered IPA produced comparable urine levels and gave a very similar metabolic picture. These results indicate that IPA is rapidly metabolized in the body, but not to uric acid, as is found for common purine nucleosides. Incubation of N^6-(Δ^2-isopentenyl)adenine with xanthine oxidase resulted in rapid oxidation, presumably, to 2,8-dihydroxy-N^6-(Δ^2-isopentenyl)adenine. On the basis of these findings *in vivo* and *in vitro*, a catabolic pathway for IPA is suggested.

IN A CONTINUING investigation of the biological activities and metabolism of the modified nucleosides[1,2] of transfer ribonucleic acid (t-RNA), this paper describes the metabolism of one such nucleoside, N^6-(Δ^2-isopentenyl)adenosine (IPA) in man.* IPA was first isolated from an enzymatic hydrolysate of t-RNA from yeast and other organisms.[3,4] Simultaneously, it was shown to be an anticodon-adjacent nucleoside in yeast t-RNA^{ser} I and II[5] and also in t-RNA^{tyr}.[6] The free base N^6-(Δ^2-isopentenyl) adenine[7] and the nucleoside IPA are very potent cytokinins.[8–10] In contrast to this, Grace *et al.*[11] found that IPA inhibited the growth of human leukemic myeloblasts (RPMI-6410) in tissue culture. Cultured lymphocytes stimulated by phytohemagglutinin were also inhibited by IPA.[12,13] However, in analogy to cytokinin activity, the lower concentrations of IPA (10^{-7} M) had a stimulatory effect on the same lymphocytes.[13] Based on the findings of Grace *et al.*[11] and the toxicological studies by Suk *et al.*,[14,15] the compound underwent clinical trial.[16] Initial results[17] indicated that very little unchanged IPA was excreted in urine, and no significant rise in urinary uric acid levels of the patients occurred. Since it is known that adenosine and guanosine terminate into uric acid in man, it was indicated that the metabolism of IPA was significantly different from the more common purine nucleosides. These observations warranted the investigation of the metabolic fate of IPA in man.

*Preliminary results of this study were presented at the 59th meeting of the American Association for Cancer Research; *Proc. Am. Ass. Cancer Res.* **9**, 13 (1968).

MATERIALS AND METHODS

Materials

All solvents used were either reagent grade or freshly distilled. Celite 545 (Johns–Manville Company), used in partition chromatography, was processed according to the procedure described earlier.[1] IPA-8-^{14}C, and a sample of 6-*N*-(3-methyl-3-hydroxybutylamine)purine were obtained from Dr. R. H. Hall. IPA and IPA-8-^{14}C were each purified to homogeneity by partition column chromatography.[3] The non-radioactive IPA* used was prepared by Starks Associates, Inc., Buffalo, N.Y. This material was purified by crystallization from isopropanol, in order to remove adenosine and other impurities. Xanthine oxidase (milk) was purchased from Worthington Biochemical Corp. (activity 0·1 unit/mg of protein; 1 unit forms 1 μmole urate from hypoxanthine/min at 25°).

Methods

Chromatrography. The following solvents were used for paper chromatography: (A) isopropanol–conc. ammonium hydroxide–water (7:1:2); (B) isopropanol–conc. hydrochloric acid–water (680:170:144); (C) isopropanol–1% aqueous ammonium sulfate(2:1);(D) dimethylformamide–chloroform–water (50:40:10);(E) ethyl acetate–2-ethoxyethanol–16% formic acid (4:1:2); (F) 1-butanol–conc. ammonium hydroxide–water (86:14:5); (G) ethyl acetate–*n*-propanol–water (4:1:2); (H) *n*-propanol–conc. ammonium hydroxide–water (7:1:2).

Whatman No. 3 MM paper was used for the initial preparative paper chromatography of column eluates. Whatman No. 1 was used for comparative identification. All chromatograms were developed by the descending method. In all instances, appropriate authentic samples were simultaneously chromatographed in parallel lanes with the purified IPA metabolites. Chromatograms were viewed under a short-wave ultraviolet lamp. All ultraviolet-absorbing areas were eluted, and analyzed in a Cary-14 spectrophotometer.

The labeled metabolites were compared with the several known compounds with regard to their ultraviolet spectra and mobility in paper chromatography.

Determination of radioactivity

Urine samples and paper eluates were assayed for radioactivity in a Packard 307 liquid scintillation spectrometer. Generally 100 μl urine was absorbed on 1 in^2 of 3 MM Whatman paper. The paper squares were then dropped into individual vials containing 20 ml of scintillation liquid and counted. The ^{14}C determinations were carried out with 88 per cent efficiency.

Administration of IPA

All experiments involved normal human volunteers kept on a low purine diet. All subjects provided 24-hr control urines. IPA was administered intravenously and orally.

*N^6-(Δ^2-isopentenyl)adenosine is unstable at room temperature and undergoes a slow degradation to adenosine and a fluorescent compound. IPA recrystallized twice from isopropanol (300 ml/10 g of IPA), did not show more than a faint trace of impurity in paper chromatography in solvent A. When stored in dark at −90° under nitrogen, the material is stable for 8 weeks.

Intravenous. To 2·0 g IPA dissolved in 25 ml of hot absolute ethanol was added 6×10^6 counts/min of IPA-8-^{14}C. This solution was then introduced into 1000 ml of sterile 5% dextrose in isotonic saline. The mixture was then given intravenously to a volunteer over a 3·5 hr period. Before the infusion, the subject emptied his bladder, thus providing an additional control sample. Immediately after completion of the infusion, the first urine sample was collected. Thereafter the urine samples were collected as recorded in Fig. 1.

Oral. To 500 mg IPA dissolved* in 150 ml of orange juice mixture (1:4) was added 3×10^6 counts/min of IPA-8-^{14}C. This mixture was given to a volunteer in one dose. The bladder was emptied immediately before oral administration of the compound. Urine was collected at intervals, as noted in Fig. 2.

Administration of non-radioactive IPA

To one volunteer,† 1 g IPA (15 mg/kg) suspended in 100 ml of orange juice was given as one dose. To another volunteer, 3 g IPA was also given as a suspension in orange juice as a single dose. Bladders were emptied as before, and urine was collected as usual.

Incubation of N^6-(Δ^2-isopentenyl)adenine, IPA and 6-N-(3-methyl-3-hydroxybutylamino purine with xanthine oxidase

These compounds were incubated with xanthine oxidase (milk). To 3 ml of $3{\cdot}5 \times 10^{-5}$ M (7·1 μg/ml) isopentenyladenine in 0·05 M, pH 7·5, phosphate buffer was added 430 μg (0·043 unit) of protein (milk xanthine oxidase) in 50 μl of the same buffer. Ultraviolet spectra were recorded at room temperature immediately for zero time, and then at various time intervals (Fig. 6). Blank cuvettes contained buffer and enzyme, but no substrate. Similarly, 6-*N*-(3-methyl-3-hydroxybutylamino)purine and IPA were incubated with xanthine oxidase.

Isolation of IPA and methylated xanthines

Urine, in 250-ml portions, was extracted three times with equal volumes of ethyl acetate, and the combined extracts were evaporated to dryness (Fig. 5). The residue was subjected to fractionation on a Celite partition column,[1] followed by paper chromatography, in order to isolate IPA and other metabolites soluble in ethyl acetate.[3] In a model experiment, 5 mg IPA was applied to a Celite column and 3·3 mg (66 per cent) was recovered.

Isolation of uric acid

After extraction with ethyl acetate, the aqueous portion of the urine was adjusted to pH 2 and then cooled overnight at 4° to precipitate uric acid (Fig. 5). The fine precipitate was collected by pouring off the supernatant and centrifuging the residual aqueous fluid. The material was recrystallized twice from boiling water, and was isolated as a white crystalline solid. Its melting behavior was identical to that of authentic sample (i.e. decomposed without melting). This material was found to be homogeneous by paper and thin-layer chromatography. Its ultraviolet spectra were

*For this experiment, IPA was first dissolved in ethanol and was then added to orange juice.

†All volunteers receiving IPA orally complained of anorexia and nausea for 4 hr after ingestion. There was no vomiting or diarrhea.

identical to those of the authentic sample. The radioactivity of uric acid was determined by first dissolving it into dilute alkali and then absorbing this solution into 1 in^2 of the 3 MM Whatman paper.

Isolation of IPA metabolites

After gross radioactive analyses were complete, appropriate urine samples were pooled. The acidified urine (pH 2 supernatant) was passed through a column of Dowex-50W H+ (200–400 mesh; 30 cc of wet resin/100 ml of urine) and was eluted with 4 l. water. The resin-bound components were released by washing the column with 1 N NH_4OH (Fig. 5). This ammonia solution was concentrated at room temperature, then lyophilized to a brown mass, and finally fractionated on a Celite partition column with a 4:1:2 ethyl acetate–2-ethoxyethanol–formic acid gradient according to our procedure described previously.[1]

RESULTS

Figure 1 represents the urinary radioactivity excretion pattern of a normal human subject after intravenous administration of labeled IPA. Of the administered counts, more than 50 per cent was excreted in the first 4 hr in urine. A total of 82 per cent of the administered radioactivity was excreted over a period of 32 hr. Attempts to determine blood levels of IPA, using 25-ml aliquots of blood taken when half of the dose had been infused, failed. This failure was due partly to rapid metabolism and partly to the limitations of the analytical methods, which could not detect microgram levels of IPA in blood.

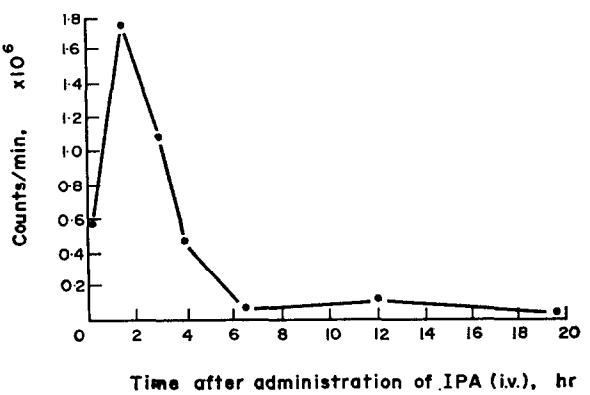

FIG. 1. Pattern of excretion of radioactivity in urine after the intravenous administration of 8-^{14}C-IPA (2 g, with 6 × 10^6 counts/min). The total counts per minute excreted at each time interval were plotted against the number of hours after administration.

Figure 2 represents the urinary radioactivity excretion pattern of a normal human subject after oral administration of 8-^{14}C-IPA. In this case, 57 per cent of the radioactivity was excreted in the first 6 hr. A total of 79 per cent was excreted over a period of 36 hr.

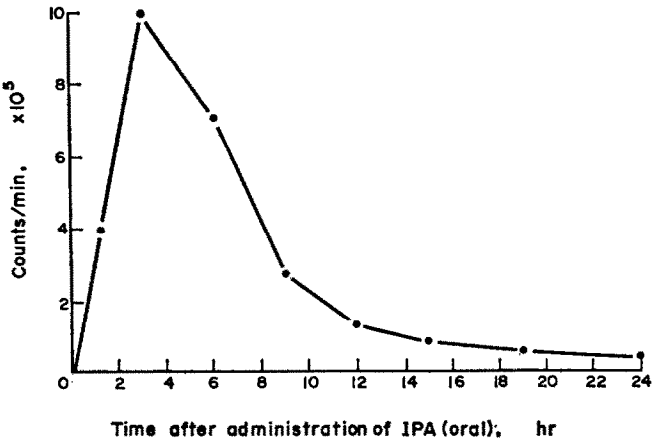

FIG. 2. Pattern of excretion of radioactivity in urine after oral administration of 8-^{14}C-IPA, (500 mg, with 3 × 10^6 counts/min). The total counts per minute excreted at each time interval were plotted against the number of hours after administration.

Figure 3 shows a profile of radioactive and ultraviolet-absorbing substances obtained through a partition chromatography of the ammonia eluate of the Dowex-50-W H+ column. Most of the radioactivity was eluted in the first 600 ml of the solvent. Another portion of the radioactivity was eluted in the fractions from 1100 to 1600 ml. A small portion was also obtained in the final 1 N NH_4OH wash of the column. These fractions were lyophilized and were purified by repeated paper chromatography until each metabolite was chromatographically homogeneous with constant specific activity. The ultraviolet spectra (Table 1) and paper chromatographic comparison

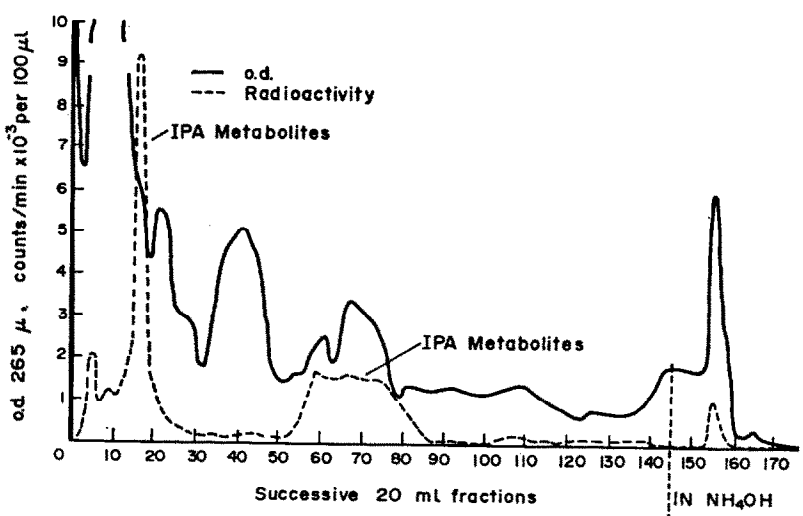

FIG. 3. Fractionation of ammonia wash on a Celite partition column. The column was eluted with a linear gradient (atmospheric pressure) of EtoAc–2-ethoxyethanol-4% HCOOH (4:1:2); EtoAc–2-ethoxyethanol–16% HCOOH (4:1:2) (1600 ml of each). Finally the column was washed with 1 N NH_4OH.

TABLE 1. COMPARISON OF ULTRAVIOLET SPECTRAL MAXIMA OF THE IPA METABOLITES WITH THE AUTHENTIC SAMPLES

Compound	Acid (pH 1–2)	Neutral (pH 6–6·5)	Base (pH 11·5–12)
IPA			
Standard	264	268	268
Metabolite	264	267	268
N^6-(Δ^2-isopentenyl)adenine			
Standard	273	269	275 282 Sh*
Metabolite	273	268	275 282 Sh*
6-*N*-(3-methyl-3-hydroxybutylamino)purine			
Standard	271	267	274 282 Sh*
Metabolite	268	268	273 282 Sh*
Adenine			
Standard	263	261 268 Sh*	269 277 Sh*
Metabolite	263	261 268 Sh*	269 277 Sh*
Hypoxanthine			
Standard	247	249	260
Metabolite	248	249	261

*Sh = shoulder.

with authentic samples (Table 2) led to identification of 6-*N*-(3-methyl-3-hydroxybutylamino)purine (II), hypoxanthine (V), adenine (IV) and some N^6-substituted adenines and *N*-alkylated xanthines (Table 3 and Fig. 4). Uric acid (VI) isolated by precipitation from the urine, was purified by crystallization. It had negligible radioactivity. Flow diagram in Fig. 5, shows the percentage recovery and the losses of the

TABLE 2. COMPARISON OF CHROMATOGRAPHIC MOBILITIES OF THE IPA METABOLITES WITH THE AUTHENTIC MATERIALS

Compound	($R_f \times 100$ in various solvents)							
	A	B	C	D	E	F	G	H
IPA								
Standard	83	76			75	80	91	
Metabolite	83	76			74	80	92	
N^6-(Δ^2-isopentenyl) adenine								
Standard	87	75			76	85	93	
Metabolite	87	75			76	85	93	
Adenine								
Standard	37	28	52	13	27	32	34	52
Metabolite	37	29	53	13	27	32	35	54
Hypoxanthine								
Standard	31	23	51		20	09	21	55
Metabolite	32	22	50		20	09	21	55
Uric acid								
Standard	17	25	32	60				
Metabolite	18	25	33	62				
6-*N*-(3-methyl-3-hydroxybutylamino purine								
Standard	77	55			46	74	78	
Metabolite	77	54			47	73	77	

TABLE 3. URINARY METABOLITES ISOLATED FROM URINE COLLECTION AFTER IPA ADMINISTRATION (i.v.)

Compound	% of Radioactivity administered	% of Total radioactivity excreted	% of Total pure and impure radioactivity isolated
IPA	0·25	0·30	0·93
N^6-(Δ^2-isopentenyl)adenine	0·056	0·066	0·20
6-*N*-(3-methyl-3-hydroxybutyl)aminopurine	0·420	0·506	1·57
Adenine	0·071	0·083	0·257
Hypoxanthine	0·050	0·059	0·18
Uric acid	0·006	0·007	0·022
1-Methylxanthine, theophylline, theobromine, 1·7-dimethylxanthine	0·0008	0·0009	0·003
Pure non-ultraviolet-absorbing compounds	9·90	11·6	35·89
Impure non-ultraviolet-absorbing compounds	16·7	19·7	60·95
Total	27·453	32·322	100

excreted radioactivity at various stages of separation of IPA metabolites. Table 3 shows the quantitative estimation of these compounds, after final purifications.

Unchanged IPA was recovered to the extent of 0·25 per cent; most of it (0·21 per cent) by extraction of the urine samples with ethyl acetate, followed by the paper chromatographic purification. Some that remained in the urine was isolated by

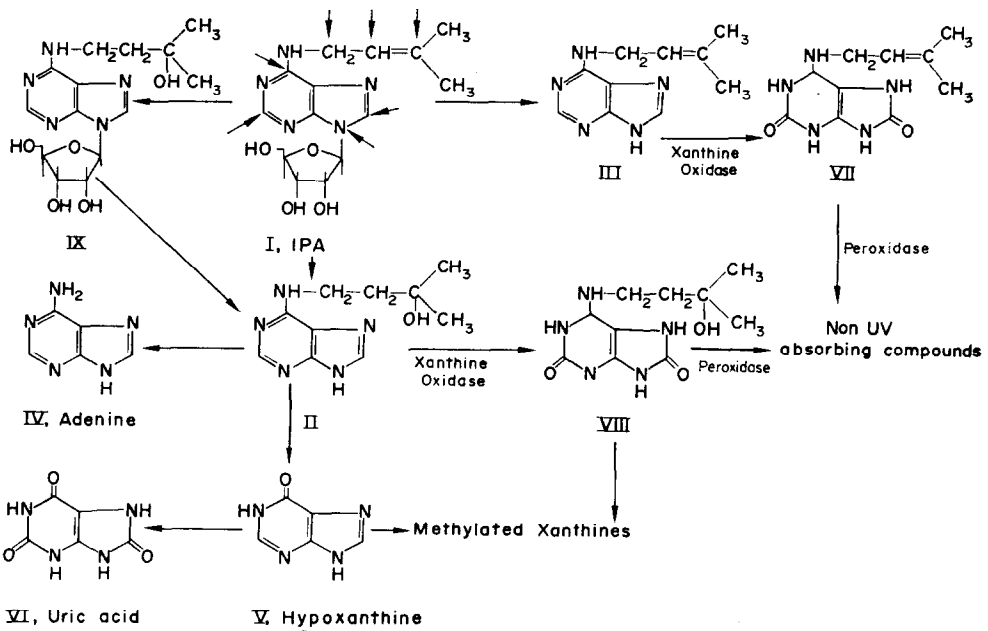

FIG. 4. Postulated metabolic pathways of IPA in man.

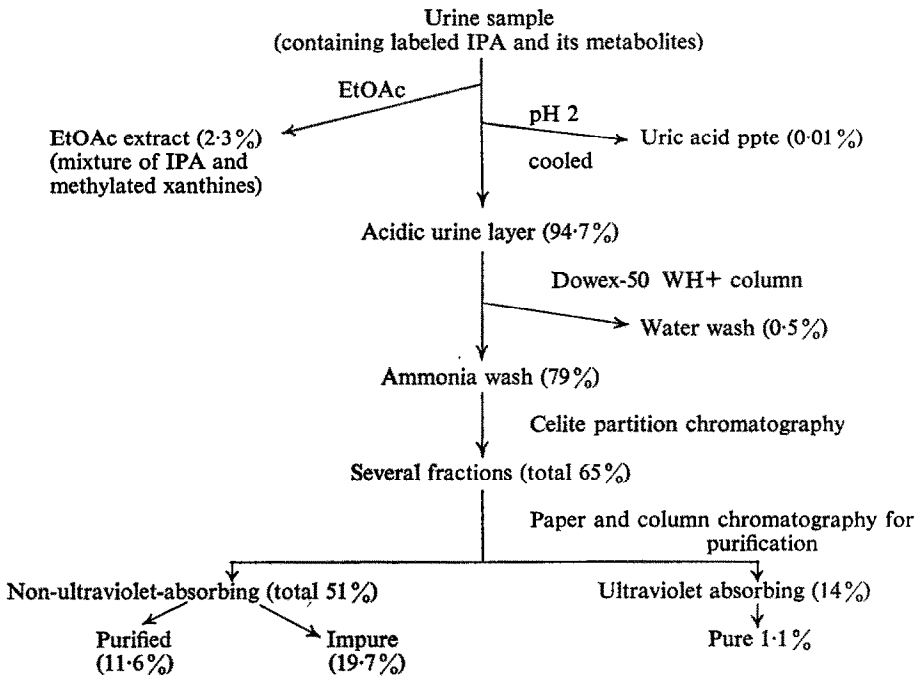

FIG. 5. Flow diagram showing the recovery and losses of radioactivity at various stages of isolation of IPA metabolites from the urine collected after the administration of 6×10^6 counts/min of IPA (2 g). Total counts per minute excreted was 82 per cent of all the administered counts. All the percentage figures shown in the flow chart are calculated on the basis of total excreted radioactivity. Losses of about 15 per cent occurred on each Dowex-50W H+column as well as on the Celite column. Much larger losses occurred, however, in purifying the materials to homogenous samples by paper chromatography.

partition column chromatography. The free base of IPA was probably formed as an artifact on the Dowex-50W H+ column, the acidic resin cleaving IPA to its free base. The compound, 6-*N*-(3-methyl-3-hydroxybutylamino)purine, hydrated base of IPA, was isolated in a relatively larger amount, and was the major ultraviolet-absorbing metabolite of IPA. Adenine, hypoxanthine and uric acid were formed as the minor metabolites. Initial evaluation had shown that about 51 per cent of the excreted radioactivity was in the form of non-ultraviolet-absorbing materials and about 14 per cent in the form of ultraviolet-absorbing metabolites. However, due to the heavy losses in the process of purification of each compound, only 12 per cent was recovered as the pure non-ultraviolet-absorbing radioactivity, while an additional 19 per cent of non-ultraviolet labeled material could not be purified further. The purification of ultraviolet-absorbing radioactivity of 14 per cent also resulted in heavy losses, finally yielding only 1·1 per cent of the labeled materials. These losses here are partly due to the contamination of the ultraviolet-absorbing compounds by lot of non-ultraviolet-absorbing material.

With the administration of IPA, excretion of methylated xanthines was greater than in control urine, but there was only a small quantity of radioactivity associated with these compounds.

Orally administered IPA was absorbed from the gastrointestinal tract and was found in urine within 2 hr after ingestion. When 1 g IPA was given orally, 1·8 mg was found in the urine in the first 5 hr. Urine samples subsequent to this period did now show free IPA. Some counts were excreted after 24 hr, in the case of orally administered radioactive IPA.

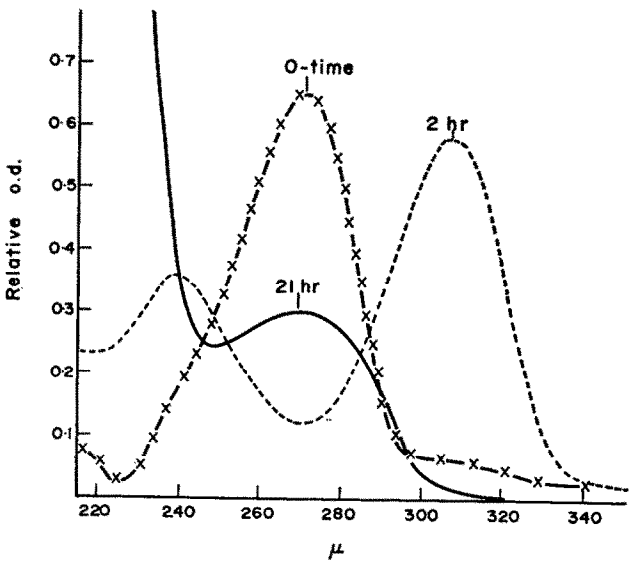

FIG. 6. Incubation of N^6-(Δ^2-isopentenyl)adenine with xanthine oxidase. Zero time, x-x-x-; 2 hr,- - -; 21 hr, ———.

Incubation of N^6-(Δ^2-isopentenyl)adenine with xanthine oxidase at pH 7·5 (Fig. 6) shows formation of a new peak at 308 mμ and disappearance of the original absorption at 262 mμ. The peak at 308 mμ is presumably due to the formation of 2,8-dioxo-N^6-(Δ^2-isopentenyl)adenine. Later, absorption at 308 mμ develops gradually over a period of 24 hr. About a third of the absorbance was left at 308 mμ. Incubation of 6-*N*-(3-methyl-3-hydroxybutylamino)Purine, II, with xanthine oxidase gave similar results. The nucleoside IPA was inert to xanthine oxidase and adenosine deaminase.

DISCUSSION

From the results obtained, it is clear that IPA is rapidly catabolized, and that most of the labeled metabolites are excreted in the first 4–5 hr. No unchanged IPA can be detected in the urine 5 hr after oral administration. With present analytical methods, no blood levels could be determined after oral or intravenous administration of IPA.

Though IPA occurs naturally as a part of the t-RNA polymer,[4–6] it would be released as a mononucleotide only in very small amounts during t-RNA turnover.[1,18,*]

* If 1615 mg t-RNA is catabolized per day in accordance with our calculations,[17] the amount of IPA liberated in 24 hr would be 0·81 mg (calculated on the basis of IPA moles per cent in human liver t-RNA 0·05, mol. wt. 335). However, IPA has not been detected in the urine of normal human subjects.[19]

Thus intravenous or oral IPA (40 mg/kg) may be metabolized in a nonspecific manner by a group of drug-metabolizing enzymes,[20] and not necessarily by IPA specific enzymes.[21] Exogenous IPA is not incorporated as such into t-RNA; its biosynthesis occurs at a macromolecular level.[22,23]

On the basis of structural considerations, the sites at which drug-metabolizing enzymes could attack the IPA molecule are those indicated with arrows on structure I (Fig. 4). The lipophilic isopentenyl side chain of IPA could be expected to be converted into a hydrophilic entity for the purposes of excretion, as was found in the case of the metabolism of 1-hexyl-3,7-dimethylxanthine.[24] Thus IPA is most likely first hydroxylated, giving hydrated IPA (IX, Fig. 4), and then undergoes sugar cleavage, giving the free base II. Hydroxylation is very commonly encountered in the metabolism of many compounds. Hydration of a double bond is rare; however, there is a precedent of this phenomenon in the tricarboxylic acid cycle (e.g. the conversion of fumarate into malate). *N*-dealkylation of this hydrated IPA base II would lead to adenine (IV). Hypoxanthine, isolated as a metabolite, could be formed from adenosine or inosine, but these were not detected. Very low levels of radioactivity were associated with 7-methylxanthine and 1,7-dimethylxanthine. There was also a low level of radioactivity with N^6-alkylated adenine (tentatively identified from ultraviolet spectra). It is well known that common purine nucleosides like adenosine and guanosine are excreted as uric acid, but only traces of uric acid were formed from IPA. These findings suggest that the metabolism of the modified nucleosides is quite different.

The salient observation in this study is that the major portion of the ^{14}C radioactivity is excreted in non-ultraviolet-absorbing compounds. There is a precedent for this type of degradation phenomenon in 6-substituted purine bases. Lettré *et al.*[25] have reported that *N*-(6-purinyl)histamine, a potent cytotoxic compound to tumor cells in tissue culture,[26] is oxidized by xanthine oxidase to 2,8-dihydroxy *N*-(6-purinyl)histamine,[25] which then readily degrades in the presence of peroxidase to non-ultraviolet-absorbing compounds.[27] In analogy to the case of *N*-(6-purinyl)-histamine,[25] incubation of N^6-(Δ^2-isopentenyl)adenine with xanthine oxidase led to the formation presumably of the 2,8-dihydroxy product, VII, which on prolonged incubation underwent degradation to non-ultraviolet-absorbing material. Similar results were obtained with 6-*N*-(3-methyl-3-hydroxybutylamino)purine, II, on incubation with xanthine oxidase. Thus non-ultraviolet-absorbing material can be formed from IPA via the free base or the hydroxy compound II. Oxidation and ring opening in the pyrimidine and imidazole moieties must occur if the product is to be non-ultraviolet-absorbing. Accordingly, it is possible that the compound is first oxidized at the 8- and 2-positions of the purine nucleus, and then undergoes cleavage leading to the non-ultraviolet-absorbing material. Study of this material indicates that it does not consist of hydantoin or ureido compounds.

Studies in perfused rat liver show that here also IPA is metabolized to non-ultraviolet-absorbing compounds and that no unchanged IPA could be detected after 2 hr of perfusion.* Analysis of the perfused plasma subsequent to 4 hr showed a majority of the counts to be in the non-ultraviolet-fraction. Similarly, incubation of IPA with chicken bone marrow led to rapid degradation to 6-*N*-(3-methyl-3-hydroxybutyl-amino) purine and non-ultraviolet-absorbing materials.[21]

*G. B. Chheda and A. Mittelman, unpublished observation.

The exact mechanism of antileukemic action of IPA in man[16] still remains unknown. In preliminary studies, IPA has been shown to interfere with methionine metabolism in cultured mammalian cells.[28] It has also been observed that IPA markedly inhibits RNA synthesis in human lymphocytes.[13]

Our studies indicate that IPA is rapidly catabolized in man and that only very little intact IPA may reach the target site of action. It would be of interest to see if stabilized IPA derivatives or metabolically stable analogs would show greater activity *in vitro* and *in vivo*.

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