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Metabolism of [6-^{14}C]allopurinol—Lack of incorporation of allopurinol into nucleic acids

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Allopurinol [4-hydroxypyrazolo(3,4-d)pyrimidine] is an important therapeutic agent in the management of various types of hyperuricemia, including gout [1]. Plasma uric acid levels are lowered by virtue of the inhibition of xanthine oxidase by both allopurinol and its principal metabolite oxipurinol [4,6-dihydroxypyrazolo(3,4-d)pyrimidine], which persists in the plasma because of active reabsorption by the kidney tubule [2].

The ribonucleoside-5′-monophosphate derivatives of allopurinol (Alo-5′-P) and oxipurinol (1-Oxi-5′-P and 7-Oxi-5′-P) have been synthesized enzymatically *in vitro* [3, 4] and have been found at micromolar concentrations in tissues of rats given large intravenous doses of [6-^{14}C]allopurinol [5]. These ribonucleotide derivatives, particularly 1-Oxi-5′-P, inhibit orotidylate decarboxylase *in vitro* [4], and are thought to be responsible for the orotidinuria observed in animals and patients taking allopurinol [6, 7].

Ten years of clinical experience with allopurinol and extensive biologic and metabolic studies have shown allopurinol to be a safe and effective drug for prolonged administration. Nevertheless, an important and persistent question has been whether allopurinol or one of its metabolites may be incorporated into nucleic acids.

Previous studies with [6-^{14}C]allopurinol failed to demonstrate any radioactivity in the nucleic acids of either mouse liver [8] or cultured human fibroblast cells [9]. Moreover, examination of the acid-soluble nucleotides in rat tissues after a large intravenous dose of [6-^{14}C]allopurinol had revealed no measurable levels, i.e. $<10^{-9}$ M, of pyrazolopyrimidine analogs of AMP or GMP and no pyrazolopyrimidine nucleoside di- or triphosphates [5]. Nevertheless, it was considered important to rule out the possibility of incorporation unequivocally.

A large dose, 50 mg/kg, i.v. of [6-^{14}C]allopurinol of high specific activity (2·4 mCi/mmole) was given to female Sprague–Dawley rats and several tissues were examined, including a rapidly dividing one such as intestine. The animals were sacrificed at 4 hr, a time at which previous studies [5] had indicated the presence of a maximum concentration of allopurinol and oxipurinol ribonucleotides. Samples of liver, kidney, spleen and intestine were frozen with liquid nitrogen-cooled clamps and extracted by a phenol method described by Kimura *et al.* [10]. Nucleic acids obtained from the aqueous phase and from the "interphase" were differentially precipitated with cetyltrimethyl ammonium bromide (CTAB) to separate RNA and DNA [11] and to remove adsorbed low molecular weight contaminants [12] known to be present in phenol-extracted RNA. Several cycles of precipitation with CTAB gave a preparation of nucleic acid with a constant specific activity (Table 1). Liver RNA had the lowest specific activity, 44 dis/min/mg RNA, and intestinal RNA had the highest activity, 429 dis/min/mg RNA with the RNA of kidney and spleen being intermediate. These values correlate roughly with the mitotic activity of these tissues. The total amount of radioactivity in the intestinal RNA was 0·0025 per cent of the administered dose. The DNA isolated from the rat intestine contained less than 10 dis/min/mg, so that further analytical work was impossible.

The acid-soluble nucleotides in the rat intestine were separated by the method described by Nelson *et al.* [5] and the concentrations of 1-Alo-5′-P, 1-Oxi-5′-P and 7-Oxi-5′-P were 1·3, 0·9 and 1·0 nmoles/g wet wt. These levels are of the same order of magnitude as had previously been found in liver [5]. As in the earlier work, there was no radioactivity in the ATP or GTP fractions.

Since the intestinal RNA was available in the largest quantity and had the highest radioactivity, it was selected for identification of the radioactive species present. The intestinal RNA was hydrolyzed with KOH, 0·3 M, at 22° for 18 hr and the resulting mixture of 2′- and 3′-ribonucleoside monophosphates was separated by chromatography

Table 1. Incorporation of radioactivity from [6-^{14}C]allopurinol into nucleic acids of rat tissues*

	Specific activities	
	Expt. I	Expt. II
	(dis/min/mg)	
Liver RNA	44	
Kidney RNA	140	
Spleen RNA	226	
Intestinal RNA	513	429
Intestinal DNA	<10	<10
KOH hydrolysis of intestinal RNA	(dis/min/μmole)	
2′,3′-AMP	213	231
2′,3′-GMP	463	230
	(dis/min/mg)	
Calculated sp. act. of intestinal RNA	543	451

* In Expt. I, two female Sprague–Dawley rats were given [6-^{14}C]allopurinol, 1·7 μCi/μmole, at a dose of 50 mg/kg, i.v. In Expt. II, four rats were used and the specific activity of the allopurinol was 2·4 μCi/μmole. At 4 hr the animals were sacrificed and the nucleic acids were isolated and analyzed as described in the text.

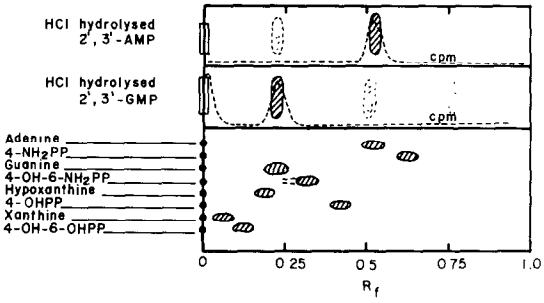

Fig. 1. Paper chromatographic separation of purine and pyrazolo(3,4-d)pyrimidine bases. The upper panel shows the HCl hydrolysis products derived from the 2',3'-AMP fraction of a digest of intestinal RNA from rats treated with [6-^{14}C]allopurinol; the middle panel shows the products from the HCl hydrolysis of 2',3'-GMP. The hatched areas indicate spots visualized with an ultraviolet lamp; the dashed line represents the tracing from a radioactivity strip scanner. The R_f values of the standards are shown in the lower panel. The solvent was *n*-butanol–conc. ammonium hydroxide–water (84:1:15, v/v). The chromatogram was run in ascending fashion overnight, using Whatman No. 1 paper. Abbreviations are: 4-aminopyrazolo(3,4-d)pyrimidine, 4-NH_2PP; 4-hydroxy-6-aminopyrazolo(3,4-d)pyrimidine, 4-OH-6-NH_2PP; allopurinol, 4-OHPP; oxipurinol, 4-OH-6-OHPP.

on a 1 × 20 cm column of Biorad AG-1 × 8 (formate) (Calbiochem, Inc.) with a gradient of 0 to 6 M formic acid [5]. All of the radioactivity which was applied to the column appeared in four distinct peaks of radioactivity which overlapped precisely with ultraviolet-absorbing (254 nm) peaks of 2'- and 3'-AMP and 2'- and 3'-GMP. Fractions containing the 2'- and 3'-AMP were pooled, as were the 2'- and 3'-GMP fractions, and the formic acid was removed by evaporation *in vacuo*. Specific activities of the 2',3'-AMP and 2',3'-GMP were determined (Table 1), based on quantification from the ultraviolet absorption spectra at pH 1. If one assumes that the purine base composition of rat intestinal RNA is similar to that of rat liver ribosomal RNA, i.e. 19 moles % adenine and 33 moles % guanine [13], it is possible to calculate the specific activity of the RNA from the measured specific activities of the 2',3'-AMP and 2',3'-GMP. Such calculated specific activities agree very well with the experimentally determined values: 543 (calc.) vs. 513 dis/min/mg (experimental) in Experiment I, 451 (calc.) vs. 429 dis/min/mg (experimental) in Experiment II (Table 1). This close agreement indicates that there was no significant loss of a radioactive component in the alkaline hydrolysis or chromatographic procedure.

It was still necessary to rule out the possibility that part or all of the radioactivity recovered in the AMP and GMP fractions was due to a corresponding pyrazolopyrimidine ribonucleotide which would elute coincidentally with the purine ribonucleotide from the column. The final step in the analysis required that the radioactive base in each case be unequivocally identified. Both the AMP and the GMP fractions were treated with HCl, 1 N, at 100° for 1 hr. Paper chromatography on Whatman No. 1 with *n*-butanol–ammonium hydroxide–water (84:1:15, v/v), using an ascending overnight development, separated all of the possible ^{14}C-labeled compounds. The unlabeled markers and their R_f values in this system were: adenine (0·53), guanine (0·22), hypoxanthine (0·18), xanthine (0·05), allopurinol (0·40), oxipurinol (0·11), 4-aminopyrazolo(3,4-d)pyrimidine (0·62) and 4-hydroxy-6-aminopyrazolo(3,4-d)pyrimidine (0·32). Only radioactive adenine was found in the 2',3'-AMP fraction and only radioactive guanine was found in the HCl-hydrolyzed 2',3'-GMP fraction (Fig. 1).

Since the known nucleoside monophosphate kinases are incapable of phosphorylating either IMP or XMP, one would not expect the higher phosphate derivatives of the analogous 1-Alo-5'-P or 1-Oxi-5'-P to be formed. Enzymatic studies *in vitro* [14] have shown that 1-Oxi-5'-P is at a greater than 10^{10}-fold kinetic disadvantage compared with XMP in the reactions leading to GTP, which is in agreement with the absence of pyrazolopyrimidine bases in the nucleic acids.

The [6-^{14}C]allopurinol used in this study was free of [^{14}C]formate and [^{14}C]hypoxanthine which, if they had been even minor contaminants, could have been the precursor of the labeled adenine and guanine reported in this study.

The most reasonable explanation for the appearance of ^{14}C in the adenine and guanine of RNA is that a small fraction of the [6-^{14}C]allopurinol undergoes a ring opening between C-5 and C-6, followed by the loss of the C-6 ^{14}C-carbon, which enters the 1-carbon pool as [^{14}C]formate. A portion of the [^{14}C]formate could easily become incorporated into the purine ring via the *de novo* biosynthetic pathway. This might have been predicted, since Elion *et al.* [8] reported that 0·06 per cent of a dose of [6-^{14}C]allopurinol (50 mg/kg) to a mouse was expired as [^{14}C]carbon dioxide during the first 2 hr. An analogous ring opening of inosinic acid has been described where the product, 5-formylamino-4-imidazolecarboxamide-1-ribonucleoside-5'-phosphate, may lose the formyl group [15]. One of the unidentified ^{14}C-metabolites of [6-^{14}C]allopurinol described by us previously [5] may be such a ring-opened compound. The excision of carbon-6 of allopurinol is known to occur chemically [16].

In agreement with the hypothesis that the ^{14}C in the adenine and guanine of the RNA came via [^{14}C]formate is the striking similarity of the results reported here to the pattern of incorporation of [^{14}C]formate into the RNA of rat tissues reported by Drochmans *et al.* [17], i.e. approximately equal radioactivity in the adenylate and guanylate moieties, and intestinal RNA much more radioactive than liver RNA. Moreover, if the labeled precursor were a preformed purine, one would expect the liver RNA to have a higher specific activity than intestinal RNA, as was found by Drochmans *et al.* [17] using [^{14}C]adenine.

In a study of this kind it is instructive to calculate the lower limit of detectability of an incorporated pyrazolopyrimidine base in the nucleic acids. If 5 per cent of the radioactivity in the intestinal RNA had been due to a pyrazolopyrimidine, i.e. 25 dis/min/mg, this amount would have been detectable on the paper chromatogram of the hydrolyzed 2',3'-AMP and 2',3'-GMP fractions. Therefore, there could have been no more than 5×10^{-6} μmoles/mg RNA or one pyrazolopyrimidine base per 5×10^6 normal bases. The DNA had less than 2 per cent of the radioactivity of the RNA. If the pattern of incorporation of [^{14}C]formate into adenine and guanine of DNA were the same as in RNA, one could estimate that the upper limit of incorporation of a pyrazolopyrimidine base into DNA would be 1 per 10^7 normal purine bases. This is less than one base substitution per entire DNA molecule of molecular weight 10^9.

The present studies confirm the lack of incorporation of allopurinol or its metabolites into nucleic acids. There is no experimental or clinical evidence to suggest that allopurinol poses a genetic hazard.

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Reduction in isoprenaline-induced cyclic AMP formation in guinea-pig heart after exposure to isoprenaline or salbutamol

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Occasionally it has been observed that prolonged treatment of a tissue (*in vitro* or *in vivo*) with a sympathomimetic amine results in a decrease in its responsiveness to subsequent applications of that, or in some cases another sympathomimetic amine [1–8]. In rat pineal gland [6], guinea-pig macrophages [7], and human embryonic fibroblasts [8] a desensitization in catecholamine-induced cyclic AMP accumulation was observed. Conversely, an increase in the formation of cyclic AMP was observed following denervation of rat pineal gland [9].

However, conflicting reports exist on the occurrence of a change in responsiveness to catecholamines following prolonged exposure to adrenergic agonists in cardiac muscle. Atkinson and Rand [2] found that during maintained infusion of adrenaline or isoprenaline in the anaesthetised cat there was a reduction in the rise in heart rate in response to subsequent doses of either amine. Similar observations were made in man and dog by Conolly *et al.* [4]. In contrast, Kingsley *et al.* [10] found that in man such a reduction only occurred because the initial heart rate was already elevated; in addition the dose of isoprenaline required to obtain maximal chronotropic effects was unchanged. Similarly, McDivitt *et al.* [11] found no evidence of resistance to isoprenaline in anaesthetised dogs. Recently it has been reported that maintained exposure of guinea-pig atria to salbutamol, soterenol, MJ7999-1, orciprenaline or terbutaline results in a competitive antagonism of the positive chronotropic actions of *l*-isoprenaline [1, 12]. In the present experiments it was attempted, therefore, to demonstrate whether a change occurs in the accumulation of catecholamine-induced cyclic AMP in cardiac muscle after exposure to isoprenaline or salbutamol.

METHODS

Guinea-pigs of either sex, weighing between 250 and 400 g were killed by a blow to the head. Each heart was collected and perfused at a rate of 6 ml/min via an aortic cannula with McEwen's solution [13] maintained at 37° and saturated with 5% CO_2 in O_2. In some experiments *d,l*-isoprenaline or *d,l*-salbutamol was added to the McEwen's solution using a motor-driven syringe. Isoprenaline was administered as single injections either alone or during the continued perfusion with isoprenaline or salbutamol at a point close to the aortic cannula in volumes of 0·5 ml over a period of 5 sec. Tissue was collected for cyclic AMP assay 15 sec after the injection of a single dose of isoprenaline or in other cases, after varying periods of perfusion. The ventricle was cut from the heart, shaken to remove excess fluid and quickly frozen in a brass clamp chilled in liquid N_2. The tissue was then weighed and ground to a fine powder under liquid N_2 and extracted with 5% trichloracetic acid. In order to determine the recovery of the nucleotide, [^{3}H]cyclic AMP (25 nCi, 0·8 pmole, Radiochemical Centre, Amersham) was added to the extract. The extract was then centrifuged at a temperature of 4° for 15 min and a force of 10,000 *g*. Subsequent procedures for the analysis of cyclic AMP were in accord with the method of Gilman [14]. In addition, an aliquot of the solution for analysis was assayed for recovery of [^{3}H]cyclic AMP. A Packard Tri-Carb Liquid Scintillation Spectrometer was employed for estimations of radioactivity.

RESULTS AND DISCUSSION

After hearts had been perfused with McEwen's solution for 10 min, cyclic AMP levels were found to be 0·23 ± 0·03 nmoles/g wet wt. Continual perfusion of hearts with 10^{-4}M salbutamol increased cyclic AMP levels by 240 per cent when hearts were assayed at 20 sec, 1 and 10 min after commencement of perfusion with the drug (Table 1). However, after perfusion for 45 min with salbutamol, levels of cyclic AMP were similar to those in control hearts. The loss of agonist activity which occurred after perfusion with salbutamol for 45 min may be explained in terms of a change in receptor availability. This may have been due to either a slowly-developing auto-inhibi-