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SOME EFFECTS OF 4-AMINOPYRAZOLO(3:4-*d*)PYRIMIDINE ON PURINE METABOLISM*

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Abstract—In tumor-bearing mice, 4-aminopyrazolo(3:4-*d*)pyrimidine (APP) produced moderate inhibition of incorporation of formate-^{14}C into soluble purines and a more marked inhibition of incorporation into polynucleotide purines (particularly those of DNA), with a concomitant accumulation of ^{14}C in serine. This compound was without marked effect on the conversion of adenine-^{14}C to nucleotides, but did inhibit incorporation of adenine into nucleic acids. Although growth of Adenocarcinoma 755 is markedly sensitive, and growth of Sarcoma 180 relatively resistant, to inhibition by APP, nucleic acid synthesis was inhibited to about the same extent in both tumors for short periods after administration of the inhibitor; however, nucleic acid synthesis in Sarcoma 180 recovered from this inhibition much more rapidly than it did in Adenocarcinoma 755. After the *in vivo* administration of APP-6-^{14}C, Adenocarcinoma 755 and host liver contained a derivative with the properties of the nucleotide of APP. Cell-free extracts of Adenocarcinoma 755 and Sarcoma 180, when incubated with APP-6-^{14}C and 5-phosphoribosyl-1-pyrophosphate, converted the labeled substrate to nucleotides, but the conversion was poor relative to that of adenine. The results point to sites of action of APP on the pathway leading to nucleic acid purines.

INTRODUCTION

4-AMINOPYRAZOLO(3:4-*d*)PYRIMIDINE (APP), an isomer of adenine first synthesized by Robins[2], produces marked biological effects in a variety of systems. Among the observed effects are hepatotoxicity in man,[3] prolongation of the lifespan of leukemic mice,[4, 5] and inhibition of the growth of (a) microbial systems,[4, 6, 7] (b) mammalian cells in culture,[8–10] and (c) various solid or ascites tumors *in vivo*.[4, 5, 11, 12] Although the effects of APP in bacteria and in mammalian cells in culture have been shown to be prevented or reversed by natural purines or related compounds,[4, 6, 10] there has been, with the exception of the recently published results of Booth and Sartorelli[13], and Henderson and Junga[14], little information about specific biochemical sites of action of this compound.

As an isomer of adenine, APP might possibly interfere with the interconversion of purine nucleotides, the synthesis of nucleic acids, or with the synthesis or function of purine nucleotide coenzymes. The present paper presents the results of studies of the effects of APP on intermediary purine metabolism and synthesis of nucleic acids and also observations on the metabolism of ^{14}C-labeled APP.

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METHODS AND RESULTS

Biological systems

Adenocarcinoma 755, because of its high sensitivity to inhibition by purine analogs, and Sarcoma 180, which is relatively insensitive to purine antagonists, were chosen as suitable biological systems for study. Adenocarcinoma 755 was grown subcutaneously in C57 black mice and used for metabolic studies 12–14 days after implantation. Sarcoma 180 was grown subcutaneously in Swiss mice and used for metabolic studies 7–8 days after implantation. All mice were adults of weight range 18–22 g and both tumors weighed approximately 1 g at the time of use. All injections, both of APP and tracers, were given intraperitoneally.

Materials

Sodium formate-^{14}C and adenine-8-^{14}C of high specific activity (1·5–5·9 mc/m-mole) were purchased from Isotopes Specialties Company. 5-Phosphoribosyl-1-pyrophosphate was obtained from Pabst Laboratories. APP labeled in the 6-position with ^{14}C was prepared from 3-amino-4-cyanopyrazole, formamide, and sodium formate-^{14}C, essentially by the procedure of Robins,[2] except that the amount of formamide was reduced to avoid excessive dilution of specific activity. The resulting product (specific activity, 0·52 mc/m-mole) was purified by reprecipitation from warm alkali and assayed for radiopurity by paper chromatography and autoradiography.

Effects of APP on incorporation of formate and adenine into soluble purines and nucleic acid purines

APP and the labeled compounds were administered to tumor-bearing animals according to the schedules given in Tables 1 and 2. The procedures for the isolation and assay of soluble purines and nucleic acid purines have been described in detail elsewhere.[15] The term "soluble purines", as used in Table 1, includes all of the soluble forms in which a given purine occurs.

From the data in Table 1 it is apparent that in tumor and intestine (a) APP markedly inhibited the incorporation of formate-^{14}C into nucleic acid purines; (b) that inhibition of incorporation into deoxyribonucleic acids (DNA) was greater than that into ribonucleic acids (RNA); and (c) that there was slight, if any, inhibition of incorporation into soluble purines. The results with adenine-^{14}C were qualitatively similar to those obtained with formate-^{14}C, but the degree of inhibition of incorporation into polynucleotides was less and there appeared to be some accumulation of radioactivity in the soluble purines. The incorporation of both precursors into some purine fractions of liver was also inhibited, but the values for the polynucleotide purines with formate-^{14}C as precursor are of doubtful significance because of the very low specific activities.

In Table 2 are presented the results of experiments in which the effects of APP on the incorporation of formate-^{14}C were studied in both Adenocarcinoma 755 and in Sarcoma 180. In both tumors, the greatest inhibition was observed 6 hr after administration of the precursor, and at this time the degree of inhbition was of the same order in both tumors. The inhibition of incorporation into DNA purines was greater than that into RNA purines. The principal difference in response between the two tumors was observed at 24 hr, at which time little effect was observed on either the DNA or the RNA of Sarcoma 180, whereas in Adenocarcinoma 755 the synthesis of DNA

TABLE 1. EFFECTS OF 4-AMINOPYRAZOLO(3:4-*d*)PYRIMIDINE ON THE UTILIZATION OF FORMATE-^{14}C AND ADENINE-8-^{14}C *in vivo**

Precursor	Tissue	Specific activities of purines of treated groups as percentages of controls						
		Acid-soluble			DNA		RNA	
		Hx	Ad	Gu	Ad	Gu	Ad	Gu
Formate-^{14}C (sp. act. 5·9 mc/m-mole)	Ad755	75(3·2)†	78(2·5)	90(1·8)	15(0·09)	21(0·12)	49(0·49)	41(0·33)
	Intestine	130(4·8)	92(2·4)	120(1·4)	34(0·28)	42(0·32)	66(0·93)	58(0·64)
	Liver	63(0·34)	56(0·18)	39(0·08)	53(0·04)	52(0·03)	53(0·04)	—
Adenine-8-^{14}C (sp. act. 1·5 mc/m-mole)	Ad755	130(6·5)	125(7·8)	153(0·75)	26(0·25)	42(0·023)	70(1·4)	88(0·10)
	Intestine	144(14·0)	132(13·0)	—	61(0·72)	124(0·11)	93(3·0)	160(0·31)
	Liver	185(11·7)	168(10·1)	—	50(0·17)	—	163(1·86)	—

* Each treated animal received two intraperitoneal injections (each at a level of 30 mg/kg) of APP, one 1 hr before, and one immediately before intraperitoneal administration of formate-^{14}C (10 μc/25 g body weight) or adenine-8-^{14}C (5 μc/25 g body weight). Each control and treated group represents the pooled tissues obtained from three to six animals. Animals were sacrificed 6 hr after administration of the labeled precursors.

† The values in parentheses are the observed specific activities (c/sec per μg) of the purines of the treated groups.

was still markedly inhibited. Table 2 also contains data on the effects of adenine on formate utilization; these experiments were carried out to determine if any of the effects of APP could be rationalized as resulting from a simulation of the action of adenine either in sparing formate utilization or in inhibiting *de novo* synthesis by a negative feedback action. When administered at levels the same as those of APP, adenine did not inhibit synthesis of purines *de novo*. At much higher levels a marked inhibition was observed and the extent of inhibition into RNA was about the same as that into DNA.

TABLE 2. EFFECTS OF APP OR ADENINE ON THE SYNTHESIS OF NUCLEIC ACID PURINES *de novo* AT VARIOUS TIMES AFTER ADMINISTRATION OF SODIUM FORMATE-^{14}C*

			Specific activities of purines of treated groups as percentages of controls							
			Tumor				Intestine			
			DNA		RNA		DNA		RNA	
Time	Treatment	Tumor	Ad	Gu	Ad	Gu	Ad	Gu	Ad	Gu
3	APP	Ad755	29	31	46	43	43	33	62	37
6	APP	Ad755	12	21	48	37	46	38	94	53
24	APP	Ad755	30	36	68	66	44	28	41	84
3	APP	Sa180	46	42	71	59	75	65	105	71
6	APP	Sa180	28	24	42	36	38	31	64	40
24	APP	Sa180	62	65	89	87	78	59	110	63
6	Adenine (60 mg/kg)	Ad755	103	126	113	118	106	118	100	114
6	Adenine (200 mg/kg)	Ad755	33	45	24	33	39	55	30	41

* APP and formate were administered as described in Table 1. The indicated doses of adenine were administered similarly as two injections each of 30 mg/kg, or 100 mg/kg. The times indicated in column one are the hours elapsing between the administration of formate-^{14}C and the sacrifice of the animals.

Fig. 1 presents the results of experiments undertaken to compare the rates of recovery of polynucleotide synthesis in Sarcoma 180 and Adenocarcinoma 755 from the inhibitory effects of APP. In these experiments, labeled formate was administered at times ranging from $\frac{1}{2}$ to 24 hr after administration of APP, and 2 hr were allowed for utilization of the formate. In both tumors maximum inhibitory effects were reached at about 3 hr after administration of APP. However, after this period, inhibition of both DNA and RNA synthesis was maintained for an additional 15 hr in Adenocarcinoma 755, whereas nucleic acid synthesis in Sarcoma 180 began to recover rapidly and within 12 hr had completely escaped from the inhibitory effects. Values are shown in Fig. 1 only for adenine; the values for guanine showed the same relative changes as those for adenine. Some of the specific activities for DNA and RNA adenine of the APP-treated Adenocarcinoma 755 were so low that they could not be assayed accurately and could be determined only as less than a certain value; in plotting these results, as percentages of controls in Fig. 1, the maximum value has been used.

Chromatographic study of the effects of APP on the metabolism of formate-^{14}*C and adenine-8-*^{14}*C*

Further study of the effects of APP on the metabolism of adenine and formate was carried out by the chromatographic-autoradiographic technique used earlier in these laboratories for study of the mode of action of other inhibitors.[16, 17] In these experiments, APP and the labeled precursors were administered according to the schedule

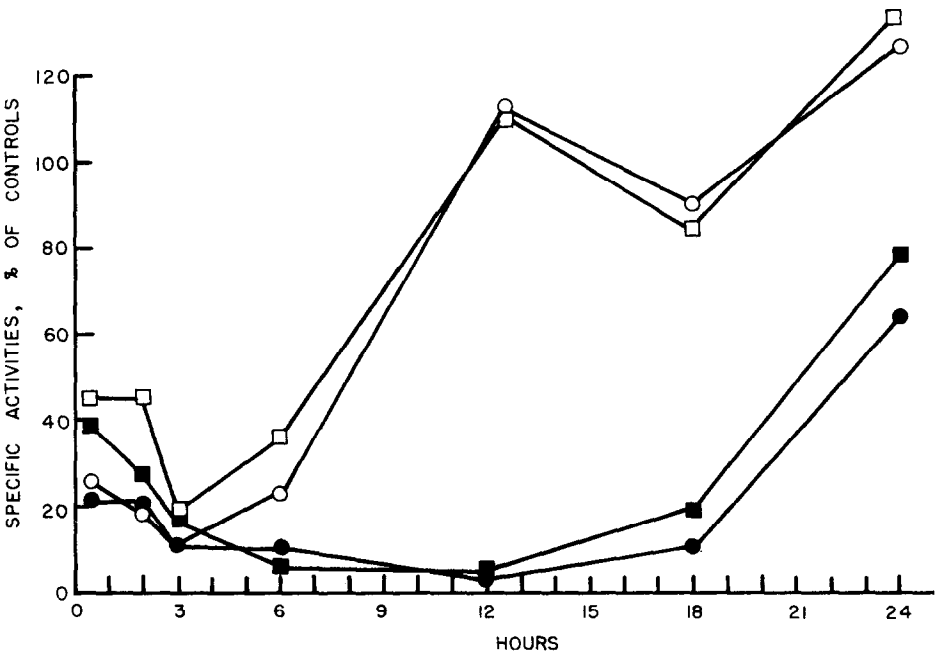

FIG. 1. Duration of the inhibitory effects of 4-aminopyrazolo(3:4-*d*)pyrimidine on the incorporation of formate-^{14}C into DNA and RNA adenine of Adenocarcinoma 755 and Sarcoma 180. ■ = Ad755 RNA; ● = Ad755 DNA; □ = Sa180 RNA; ○ = Sa180 DNA. APP was administered intraperitoneally in two divided doses, each of 30 mg/kg, 1 hr apart. The abscissae indicate the time elapsing between the administration of the second dose of APP and intraperitoneal injection of sodium formate-^{14}C at a level of 10 μc/25 g body weight. All animals were sacrificed 2 hr after administration of formate-^{14}C.

given in Table 3. Procedures for preparation of extracts of tissues, two-dimensional paper chromatography (phenol–water in one direction and butanol–propionic acid in the other), and preparation of autoradiograms from the resulting chromatograms have been described.[17] Several chromatograms were prepared from each extract.

Tentative identification of spots was made by comparison of R_f values and the general chromatographic patterns with those obtained in many similar past experiments with the same precursors.[17, 18] The filter paper areas corresponding to the various radioactive areas (as located by autoradiography) were then extracted with water, and the extracts were either counted on planchets or used for identification by paper chromatography in parallel with known compounds in appropriate solvents. In Fig. 2 are shown reproductions of autoradiograms from the experiment in which formate-^{14}C was the precursor. When either formate or adenine was the precursor, the

major part of the radioactivity was present in a spot occurring in the position of AMP. On chromatograms from both control and treated tumors this spot was identified as AMP by parallel chromatography with known AMP in three solvents; after acid-hydrolysis the radioactive material present in this spot migrated like adenine in three solvents. The other major spot on the tumor chromatograms was one tentatively identified as serine, since it moved like serine upon rechromatography in three other solvents. None of the spots on the chromatograms of liver, intestine, and spleen was identified, except by the R_f values and coincidence of chromatographic pattern with those obtained in other experiments in which exhaustive identification has been carried out. In Table 3 are presented data on the amounts of ^{14}C present in the major spots detected by autoradiography (Fig. 2).

TABLE 3. EFFECTS OF APP ON INTERMEDIARY METABOLISM OF SODIUM FORMATE-^{14}C AND ADENINE-8-^{14}C*

Precursor	Compound isolated	Radioactivity (counts/sec)							
		Tumor		Liver		Spleen		Intestine	
		C	T	C	T	C	T	C	T
Sodium formate-^{14}C (sp. act. 3·9 mc/m-mole)	AMP	391	201	84	16	171	52	247	37
	IMP + GMP	76	54	30	14	402	239	94	42
	Free bases + nucleotides					368	537	370	547
	Sum of all purine derivs.	467	255	114	30	941	826	711	626
	Serine	42	176			48	121	38	94
Adenine-8-^{14}C (sp. act. 3·5 mc/m-mole)	AMP	158	88	374	339	58	101	86	109
	ADP	10	36	43	26	7	12	22	14
	ATP	>2	9	2	3	3	3	4	5
	Sum of adenine derivs.	168	133	419	368	68	116	112	128
	IMP + GMP	20	14	43	74	65	75	28	30
	Sum of all purine derivs.	188	147	462	442	138	191	140	158

* C = control; T = treated. Control animals were given an intraperitoneal injection of sodium formate-^{14}C (100 μc/25 g body weight) or adenine-8-^{14}C (10 μc/25 g body weight). Treated animals received APP in two equal intraperitoneal injections, one 1 hr before, and one immediately before administration of labeled formate or adenine. In the groups receiving formate-^{14}C, each injection of APP was at a level of 30 mg/kg; in those receiving adenine-^{14}C each injection was at a level of 60 mg/kg. All animals were sacrificed 1 hr after administration of the labeled precursor.

From the results in Table 3 and Fig. 2, it is apparent that the major effects of APP on the metabolism of formate-^{14}C are a decreased incorporation into AMP and an increased incorporation into serine. The effects on incorporation into AMP appeared to be most profound in liver, spleen, and intestine, but in the latter two tissues formate incorporation into all purine derivatives as a group was little changed. Since some enzymic degradation may have occurred during the preparation of the tissues, the sum of the purine derivatives is a better index of the effects on the inhibition of *de novo* synthesis than are the individual nucleotides. When considered with the results in Table 1, these data show that the effects on the synthesis of purines *de novo* under these conditions are minimal and short-lived.

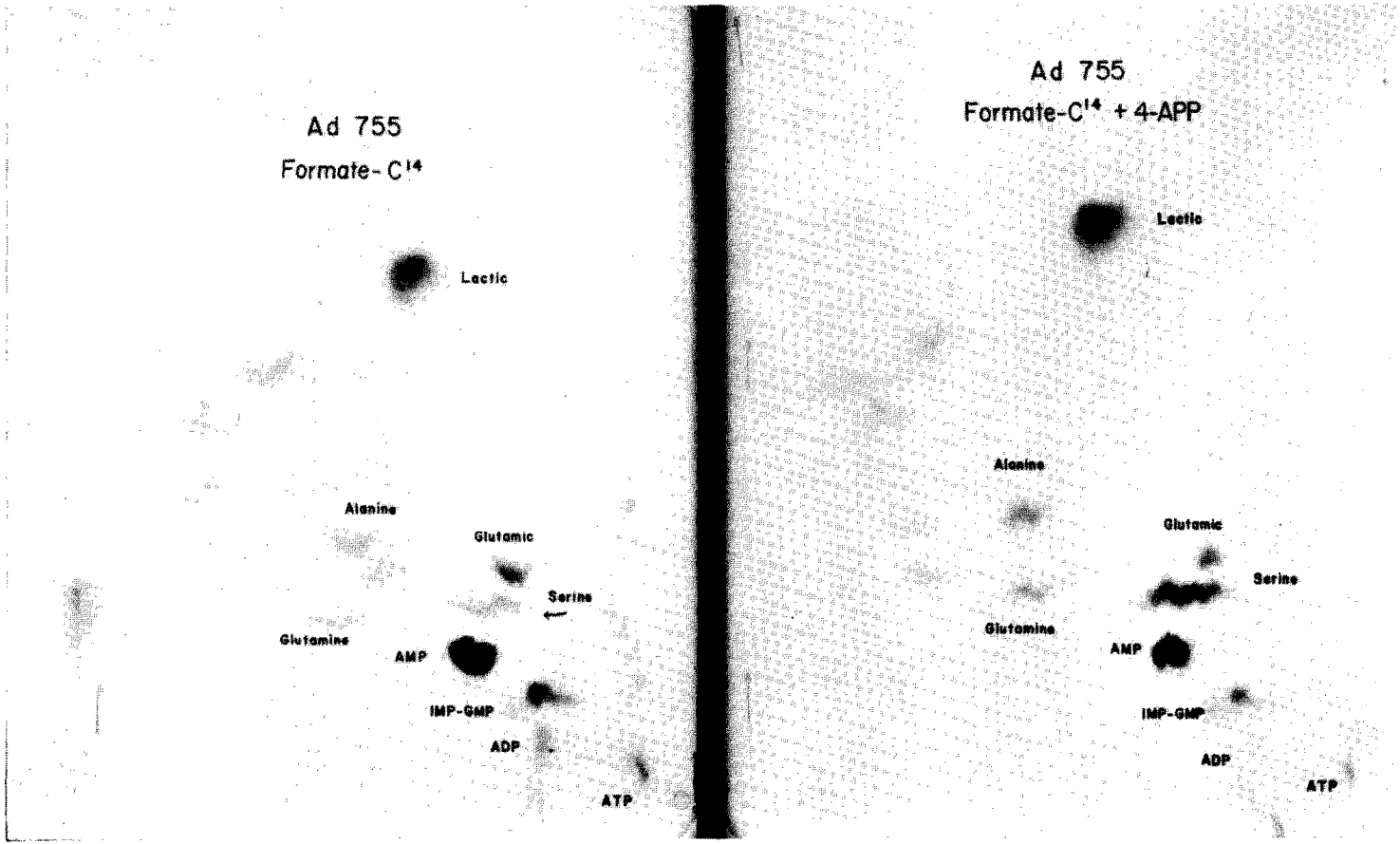

FIG. 2. Effects of 4-aminopyrazolo(3:4-d)pyrimidine on the metabolism of sodium formate-^{14}C by Adenocarcinoma 755 *in vivo*. The autoradiograms shown were prepared from chromatograms of soluble extracts obtained 1 hr after administration of formate-^{14}C. See Table 3 for experimental details and for quantitative data derived from these chromatograms.

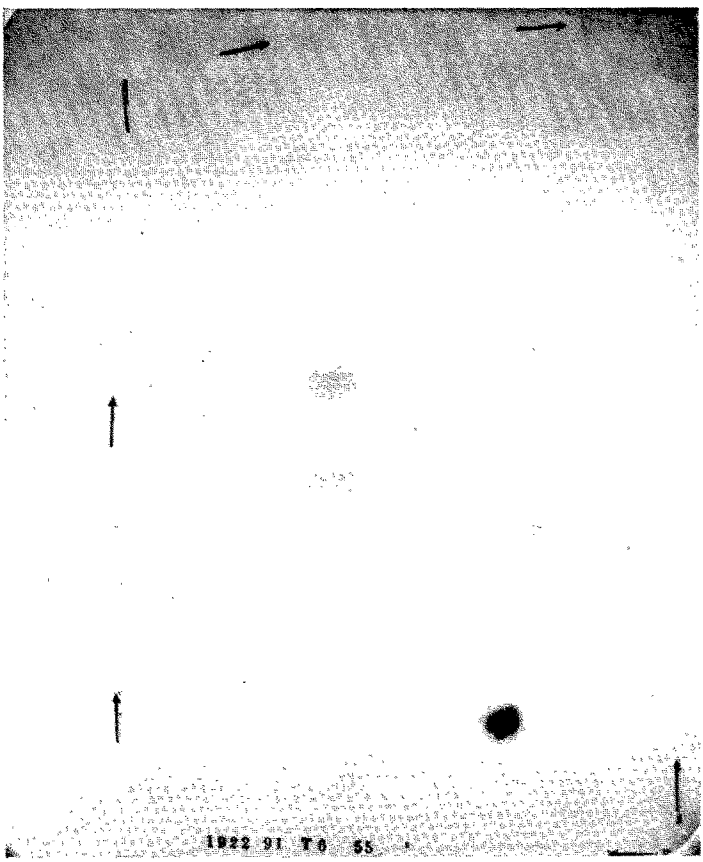

FIG. 3. Metabolism of 4-Aminopyrazolo(3:4-*d*)pyrimidine-6-^{14}C by Adenocarcinoma 755 *in vivo*. The autoradiogram shown was prepared from a chromatogram of the soluble extract obtained 6 hr after intraperitoneal administration of APP-6-^{14}C at a level of 10 μc/25 g body weight. The principal spot is the ribonucleotide of APP; the faint spot at the upper left is residual substrate. The other faint spots have not been identified.

Some changes in other less radioactive spots also occurred. Thus, compounds occurring in the positions of alanine and glutamine increased (Fig. 2), and in the liver (not shown in Fig. 2) a spot occurring in the position of allantoin decreased. A decrease in allantoin would be expected from the observation that APP and some related compounds inhibit xanthine oxidase.[19] The amount of activity in these three spots was low, however, and identification by rechromatography was, as a result, inconclusive.

The effects of APP on the metabolism of adenine-8-^{14}C (Table 3) are much less marked than those on the metabolism of formate. It is of interest that 1 hr after the administration of adenine-8-^{14}C essentially all soluble intracellular radioactivity could be accounted for in the nucleotides. No free adenine was detected by autoradiography and the bulk of the ^{14}C was present as AMP. Since some breakdown of ATP to ADP and AMP has been found to occur during chromatography, the three adenine nucleotides should be grouped together. When this is done (Table 3), it is apparent that the effect of APP on the conversion of adenine to nucleotides is minimal, the radioactivity of the adenine nucleotides of tumor and liver being 80–90 per cent of that of controls. It is apparent that there was no very profound inhibition of the incorporation of radioactivity into IMP and GMP in any tissue, although there appeared to be some stimulation of incorporation into IMP and GMP in liver and into the adenine nucleotides of spleen.

Metabolism of APP-6-^{14}C in vivo

Studies of the metabolism of this compound *in vivo* were carried out by administering the labeled material at a level of 10 μc/25 g body weight to mice bearing Adenocarcinoma 755. At periods of 1 or 6 or 24 hr after administration of the compound, the animals were sacrificed, and alcohol extracts of tumor and liver were prepared and assayed by chromatography and autoradiography as described above. A representative autoradiogram for tumor (6-hr experiment) is shown in Fig. 3. The bulk of the radioactivity is present in a spot in the nucleotide area occurring at about the same position as AMP. The other tumor and liver experiments showed the same chromatographic pattern.

The following results confirm the suspected identity of the major metabolite as the 5′-phosphate of the ribonucleoside of APP: (a) migration like AMP upon electrophoresis in ammonium formate buffer, pH 3·5, and upon chromatography in *iso*propanol in an ammonia atmosphere; (b) migration like adenosine in the *iso*propanol–ammonia solvent after the action of a crude snake venom (*Crotalus atrox*); and (c) after acid hydrolysis, migration like APP in *iso*propanol–ammonia.

While this paper was in preparation, Henderson and Junga[20] reported extensive studies on the conversion of APP to nucleotides in a number of tumors and host tissues; using ion-exchange techniques, these authors demonstrated the presence of mono-, di-, and tri-phosphates of the ribonucleoside of APP. In the present studies, little or no radioactivity was observed on the original two-dimensional chromatograms in the area in which nucleoside di- and tri-phosphates or DPN occur. However, as has already been mentioned, it is possible that enzymic action during the preparation of the tissues converted some triphosphates to monophosphates, and that the conditions of chromatography may have caused some hydrolysis of ATP. Accordingly,

it is possible that some of the isolated APP-nucleoside monophosphate was initially present as the nucleoside triphosphate.

Nucleic acids (DNA and RNA) were also isolated by salt extraction from the livers and tumors of the animals that had been sacrificed either 1 or 6 or 24 hr after administration of APP-^{14}C. Gas phase counting[21] of the carbon dioxide resulting from the oxidation of the crude nucleic acids showed the presence of very small amounts of ^{14}C. To obtain information on the chemical form of this radioactivity, a sample of RNA (from the 24-hr experiment) was hydrolyzed with acid in the presence of carrier non-radioactive APP, after which the free bases (adenine, guanine, and APP) were separated by paper chromatography. The areas of paper containing adenine, guanine, and APP were eluted exhaustively and the eluates were assayed for ^{14}C. Most of the ^{14}C was present in the eluates of the adenine and guanine bands; a small amount was present in the eluates of the APP band, but the amount was so small that it could not be certain that this did not represent contamination from the neighboring adenine band. The fact that both adenine and guanine were labeled might suggest that the labeling resulted from a trace impurity, which, since formate-^{14}C was the starting material, served as a source of a one-carbon fragment. However, it is also not unlikely that the ^{14}C incorporated into adenine and guanine arose from degradation of APP-6-^{14}C, since this compound was labeled in the position corresponding to the 2-position of adenine and it is well known that purines may lose the 2-carbon atom as a 1-carbon unit. On the whole, although these experiments do not provide conclusive evidence concerning the incorporation of APP into polynucleotides, they do show that any such incorporation, if it occurs, is very small.

Metabolism of APP-6-^{14}C in minces

Minces of Adenocarcinoma 755 and liver in Krebs-Ringer phosphate buffer supplemented with ATP and glucose were incubated with APP-6-^{14}C (0·50 μc/g of tissue) under an oxygen atmosphere for 4 hr, after which the minces were extracted and the extracts assayed by our usual chromatographic procedures. In contrast to the results of the studies *in vivo* discussed above, the area of the chromatogram corresponding to the position of AMP had, at best, amounts of ^{14}C that were barely detectable by autoradiography. Many past experiments have shown that, under the same conditions, minces of these tissues convert adenine extensively to nucleotides.

Metabolism of APP-6-^{14}C in cell-free systems

Sonicates of Adenocarcinoma 755 and Sarcoma 180 in 0·1 M Tris buffer, pH 7·6, were centrifuged at 95,000 g at 4 °C, after which the supernatant fractions were removed and portions of the crude enzyme mixture were incubated with 5-phosphoribosyl-1-pyrophosphate and adenine-8-^{14}C or APP-6-^{14}C under the conditions described in Table 4, which are those that have been used for the study of other purine nucleotide pyrophosphorylases.[22] The protein was denatured by heat and removed by centrifugation, after which the reaction mixture was analysed for content of free base and nucleotides by paper chromatography.[22] Adenine was used as a positive control, since it is known that adenine and APP are converted to nucleotides by the same pyrophosphorylase.[23]

The pyrophosphorylase activities of the supernatant fractions of the tumor are presented in Table 4. With adenine as substrate, the crude enzymes from both Adenocarcinoma 755 and Sarcoma 180 formed two ribonucleotides, identified as AMP and

IMP (Table 4). Since adenase is virtually absent from mammalian cells, the IMP probably was derived from AMP, and therefore the sum of the amounts of AMP and IMP formed may be considered a measure of the activity of adenylic pyrophosphorylase. Both enzyme preparations also converted APP-^{14}C to two nucleotide spots; the chromatographic and electrophoretic data obtained (Table 4) are consistent with the identity of these nucleotides as the pyrazolopyrimidine analogs of AMP and IMP. For the reasons already discussed, it is probable that the analog of IMP arose from deamination of APP-nucleotide, and that the sum of the amounts of these two nucleotides is a measure of the capacity of the system to form APP-nucleotide.

TABLE 4. CONVERSION OF 4-AMINOPYRAZOLO(3:4-*d*)PYRIMIDINE AND ADENINE TO NUCLEOTIDES BY SOLUBLE ENZYME FRACTIONS IN THE PRESENCE OF 5-PHOSPHORIBOSYL-1-PYROPHOSPHATE*

Enzyme source	Substrate	Characterization of Nucleotides†			mμmoles Nucleotide per mg protein in one hr
		R_f value	Relative electrophoretic migration	Product of acid-hydrolysis	
Ad755	APP-6-^{14}C	0·18	33	APP	15
		0·13	100	HPP‡	15
	Adenine-8-^{14}C	0·17	35	Adenine	270
		0·09	94	Hypoxanthine	90
Sa180	APP-6-^{14}C	0·20	38	APP	75
		0·16	99	HPP	15
	Adenine-8-^{14}C	0·17	35	Adenine	50
		0·10	98	Hypoxanthine	300

* The base (0·5 μmole, 0·2 μc), 5-phosphoribosyl-1-pyrophosphate (2 μmoles) and the crude enzyme were incubated 1 hr at 37° in Tris buffer, pH 7·5. Protein was determined by the Oyama–Eagle modification[32] of Lowry's method.

† The R_f values are the results of one-dimensional chromatography in 70% *iso*propanol in an ammonia atmosphere. Electrophoresis was carried out in ammonium formate buffer, pH 3·5; the reported values are relative to IMP as 100. The determined values for known AMP and IMP were respectively: R_f values, 0·18, 0·12; relative electrophoretic migration, 38, 100. For identification of hydrolysis products, the nucleotides were hydrolysed with 1 N HCl at 100° for 1 hr and the hydrolysate was chromatographed in parallel with known compounds in *n*-butyl alcohol saturated with 1 N NH_4OH.

‡ HPP = 4-hydroxypyrazolo(3:4-*d*)pyrimidine.

From the data in Table 4 it is apparent that enzyme preparations from both tumors converted adenine to nucleotides (AMP + IMP) to about the same extent, but that the relative amounts of AMP and IMP formed under these conditions by the two tumor extracts were quite different: the Adenocarcinoma 755 extract yielded predominantly AMP and the Sarcoma 180 extract predominantly IMP. In both tumor extracts the conversion of APP to nucleotides was very poor relative to the conversion of adenine to AMP and IMP. It is perhaps worth noting that the relative amounts of the nucleotides of APP and of 4-hydroxypyrazolo(3:4-*d*)pyrimidine found in the two systems

do not correspond to the relative amounts of AMP and IMP. This lack of correspondence is particularly striking in Sarcoma 180: whereas with adenine as substrate the AMP : IMP ratio was 0·17, with APP as substrate the ratio of APP-nucleotide to the nucleotide of 4-hydroxypyrazolo(3:4-*d*)pyrimidine was 5. It is of interest that *in vivo* (see above) no nucleotide of 4-hydroxypyrazolopyrimidine was found, APP-nucleotide being the only nucleotide detected in Adenocarcinoma 755 or in liver at either 1 or 6 or 24 hr after the administration of labeled APP. In other experiments, in which assays were made by two-dimensional chromatography and autoradiography without further identification of spots, cell-free extracts of several other types of mammalian cells were also assayed for capacity to convert adenine and APP to nucleotides under the conditions given in Table 4. Liver and spleen from normal mice, L1210 leukemic cells, and lines of L1210 resistant to 6-mercaptopurine and 8-azaguanine all showed high capacity to convert adenine to AMP and a low capacity to convert APP to nucleotides. In all cell-free systems studied the low capacity to form APP-nucleotide is in contrast to the results of the studies *in vivo* reported above and to those of Henderson and Junga[20], which showed extensive conversion of APP to nucleotides.

DISCUSSION

The observed effects of APP on the utilization of formate and adenine suggest that APP may interfere with at least two reactions in the pathway leading to nucleic acids. Thus, the moderate inhibition of incorporation of formate into soluble purine derivatives (Table 3) would reflect an inhibition at a point prior to formation of the purine ring, whereas the fact that APP inhibited the incorporation of adenine into polynucleotides without affecting its conversion to nucleotides would suggest a site of action at a point past the formation of nucleotides. A comparison of the results on incorporation of adenine and formate into both soluble purines and polynucleotides suggests that the second of these blocks is responsible for most of the inhibition of formate incorporation into nucleic acids. The inhibition of a deoxynucleotide polymerase may also be involved, since in both tumors and intestines (Tables 1 and 2) the inhibition of incorporation of precursors into DNA was greater than that into RNA.

With regard to the site of action prior to the formation of the purine ring, it appears that this blockade may not be very significant, since in the 1-hr experiments, even at the high levels of APP used, only about a 50 per cent inhibition of incorporation into nucleotides was obtained (Table 3), and in the 6-hr experiments (Table 1) the effects on incorporation of formate into total soluble purines were insignificant. Other agents, such as azaserine and amethopterin, known to block specific conversions along this pathway, cause much greater inhibition and also cause an accumulation of compounds behind the site of blockade. It is noteworthy that no intermediate along the purine pathway was observed to accumulate under inhibition by APP. The failure of an intermediate to accumulate would suggest that the site of inhibition may be prior to the entry of formate into the pathway, or that the inhibition of purine synthesis may be an effect secondary to a disturbance in some other metabolic area. Inhibition by negative feedback at an early stage of biosynthesis would appear to be a likely possibility; natural purines, particularly adenine, or their nucleotides, have been shown to inhibit by this mechanism[24–26] and the same mechanism has been suggested for various purine analogs, including APP, on the basis of their abilities to decrease the accumulation of aminoimidazole compounds by the purine-requiring *Escherichia*

coli mutant B96.[25] A feedback mechanism would explain all the results obtained on the incorporation of formate-^{14}C into soluble compounds: if the feedback inhibition were at the first step of purine biosynthesis, as observed by Wyngaarden and Ashton in pigeon liver,[26] the incorporation of formate into the purine pathway would be blocked with the result that it might be diverted into its other metabolic pathways. In fact, APP caused an accumulation of formate-^{14}C in serine; however, levels of adenine equal to those of APP gave no inhibition of incorporation of formate-^{14}C into polynucleotides (Table 2). Much higher levels of adenine did inhibit, or spare, formate-incorporation, but the effects were different from those observed with APP in that there was no selective inhibition of incorporation into DNA as compared to RNA.

These results agree with some reports on the effects of APP on synthesis of nucleic acids, but disagree with others. Henderson and co-workers[14, 27] found, in studies with ascites cells, that the effects of APP are complex; with some precursors, including glycine, marked stimulation of incorporation of isotope into polynucleotides was observed. Zimmerman *et al.*[28] observed no effect of APP on the incorporation of a number of precursors into total RNA of *Escherichia coli*. In agreement with results reported in the present paper, Booth and Sartorelli[13], working with Ehrlich ascites cells, observed a blockade of purine synthesis *de novo* at an early stage. In contrast to the present findings, these workers found evidence for a specific blockade of synthesis of polynucleotide guanine and observed no effect on the utilization of adenine as a source of polynucleotide purines. APP has also been found to inhibit protein synthesis in Ehrlich ascites cells,[13] but not in *Escherichia coli*.[28]

Studies with many other purine analogs have shown a high degree of correlation between the susceptibility of a given biological system to growth inhibition by the analog and the capacity of the system to convert the analog to nucleotides.[29, 30] In the light of these findings, it is noteworthy that, in studies with cell-free extracts, Sarcoma 180 had as much, or more, capacity to convert APP to nucleotides as did Adenocarcinoma 755. That differences were not noted in rate of nucleotide formation between these two tumors may mean simply that they do not represent a highly susceptible and highly resistant tumor pair: APP is, in fact, not completely ineffective in inhibiting the growth of Sarcoma 180. Another possible explanation is that, under *in vivo* conditions, the nucleotide may be degraded more rapidly in Sarcoma 180 than in Adenocarcinoma 755. It is also possible that APP may be inhibitory as the free base, a possibility suggested by the facts that the 1-methyl derivative of APP[4, 12, 27] and a number of 9-alkyl derivatives of 6-mercaptopurine and hypoxanthine[31] inhibit growth of tumor cells.

Finally, it should be emphasized that the results obtained, while pointing to sites of action of APP on the biosynthesis of purines and nucleic acids, do not provide information, either the one way or the other, as to whether the indicated sites of action are primary or secondary. Since nucleotides of an adenine antagonist could conceivably block any or many of the coenzyme functions of adenine nucleotides, no conclusions as to the primary site of inhibition can be drawn until studies have been carried out on the effects of APP in other metabolic areas.

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