The Metabolism of 4-Aminopyrazolo(3,4-d)pyrimidine in Normal and Neoplastic Tissues

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SUMMARY

The metabolism of 4-aminopyrazolo(3,4-d)pyrimidine (APP) has been studied in several normal and neoplastic mouse tissues.

APP is rapidly cleared from the peritoneal fluid and blood after intraperitoneal or intravenous administration. The drug is taken up by the blood cells and is lost from them less rapidly than from serum.

Approximately one-half of the administered drug is excreted in the urine from normal mice in 24 hours, and one-half of this is within the 1st hour. The rate and amount of excretion from tumor-bearing mice are much less.

The distribution of APP in the tissues was studied. Only in the tumor cells, small intestine, liver, and spleen did APP concentration increase during the first hour after administration.

The following were tentatively identified as metabolites of APP in the tissues: free APP, APP-riboside, 4-hydroxy pyrazolo(3,4-d)pyrimidine, the mono-, di-, and triphosphates of APP-riboside, and two unidentified degradation products.

Liver, spleen, kidney, small intestine, muscle, and blood were quantitatively examined for their content of these metabolites over a 2-hour period following administration of APP. In all cases the total nucleotide fraction was quantitatively most important. The free base and degradation products were also present and were especially evident in blood and muscle.

In three sensitive and four resistant tumors the predominant metabolites were the nucleotides, at all three levels of phosphorylation. In addition, small amounts of the free base and degradation products were also present.

The metabolism of APP in the sensitive tumors was characterized either by the formation of relatively very large quantities of nucleotides or by continued nucleotide formation throughout the time period studied. Resistant tumors characteristically formed relatively smaller amounts of nucleotides or degraded those initially synthesized more rapidly than did the sensitive tumors.

All tissues and tumors studied incorporated APP into their nucleic acids. No correlation was found between the rate or extent of incorporation and sensitivity to this drug. In Ehrlich ascites carcinoma there was 10 times more incorporation into the ribonucleic acid (RNA) than into deoxyribonucleic acid (DNA). APP was shown to be incorporated into this RNA in nucleotide form.

The significance of these studies of APP metabolism is discussed.

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One approach to the study of the mechanism of action of carcinostatic antimetabolites has been through investigation of their metabolism and comparison of their metabolisms in sensitive and resistant systems. Such studies not only may give clues as to the active form of a drug in the cell but also may permit deduction as to the basis of sensitivity or resistance by individual tumors to the drug. The latter type of study may have several therapeutically useful consequences. It may be possible to modify either the drug or the conditions under which it is administered in order to alter its metabolism so that a resistant tumor is rendered sensitive. It may also be possible to predict whether a tumor is resistant or sensitive to a particular drug before treatment of a patient on the basis of biochemical observations on biopsy specimens. Such examples have been shown during the studies on thioguanine by Sartorelli and LePage (20) and LePage (16). In these investigations a thioguanine-resistant subline of the sensitive Ehrlich ascites carcinoma was developed which could be made sensitive again by combination therapy with thioguanine and azaserine (26). Likewise, a test of clinical sensitivity to thioguanine has been proposed on the basis of the good correlation between the extent of incorporation of thioguanine into nucleic acids and drug sensitivity (16).

The adenine analog, 4-aminopyrazolo(3,4-d)pyrimidine, and a number of related pyrazolo(3,4-d)pyrimidines have been shown to have tumor-inhibitory activity (10, 29, 30). Studies on the mode of action of APP have recently been reported from this and other laboratories (1, 13, 25, 35), but no studies of the metabolism of this drug in vivo have yet been published. Way and Parks (38) and Roy et al. (24) have synthesized the ribonucleotide of APP with purified enzyme systems, and Bennett et al. (1) have also reported nucleotide formation by extracts of leukemia L1210 cells. The urinary excretion products of APP in the rat have been identified by Feigelson and Davidson (6, 7) and shown to be the corresponding isoguanine, hypoxanthine, and xanthine pyrazolo-pyrimidines, as well as an unidentified compound.

The nucleotide forms of other purine antimetabolites have been postulated to be the active forms of these drugs (2, 3, 21), and resistance to them has in some instances been shown to be associated with a lowered concentration of nucleotide in the cells, either through lack of pyrophosphorylase activity (2, 3), decreased permeability (25), or increased nucleotide degradation (27). It was therefore of interest to compare the rates and extent of APP-nucleotide formation in sensitive and resistant tumors. It was also of interest to determine whether any other metabolite might be found which by concentration or other indication might be suggested to be active in the carcinostatic process.

The comparison of drug metabolism in sensitive and resistant tumors should encompass more than resistant sublines developed from sensitive parent tumors, and which resemble the parent line in most biochemical respects. It is also well to study a wide variety of sensitive and resistant tumors which vary in many aspects of their metabolism to determine how many different kinds of resistance mechanisms in the area of drug metabolism may occur in nature and which might be encountered in clinical practice. The sensitive tumors used in this study were Adenocarcinoma 755 (29, 30), ascites Leukemia L1210 (29, 30), and the Ehrlich ascites carcinoma (10). The resistant tumors were Mecca ascites lymphosarcoma, 6CSHED ascites lymphosarcoma, ascites Sarcoma 180, and a thioguanine-resistant subline of the Ehrlich ascites carcinoma (Ehrlich-TG-R) which is cross-resistant to APP (10).

The incorporation of purine and pyrimidine antimetabolites into nucleic acids, leading to impaired function, has long been considered a possible mechanism by which such fraudulent bases might exert their carcinostatic or bacteriostatic action. Among the purines, 8-azaguanine has been shown to be incorporated into the nucleic acids of a wide variety of organisms and tissues, with RNA incorporation predominating (see [18] and [20] for references). Thioguanine is incorporated into both RNA and DNA in normal tissues, roughly in proportion to the rates at which guanine is incorporated into these nucleic acids (16). In both cases there is some correlation between incorporation into nucleic acids and potency in some systems. Suggestive, but less clear-cut evidence has also been adduced for incorporation of 6-mercaptopurine into nucleic acids (5, 9). The possibility that APP was incorporated into the nucleic acids of normal and neoplastic mouse tissues was therefore examined.

MATERIALS AND METHODS

APP-6-C\(^{14}\) was a gift of Dr. L. L. Bennett, Jr., Southern Research Institute, to whom we are very grateful. It was further treated in this laboratory and used at more than 99 per cent purity. This preparation had a specific radioactivity of 2000 counts/min/\(\mu\)g in our equipment. For injection it was dissolved in isotonic neutral saline. Female Swiss mice, 25–30 gm., were given in-
oculations of $1 \times 10^6$ Ehrlich ascites carcinoma cells, $5 \times 10^6$ Sarcoma 180 ascites cells, or $1 \times 10^6$ Ehrlich-TG-R cells. Female AKD2F1 mice, 16–20 gm., received $6 \times 10^6$ Mecca ascites lymphosarcoma cells; female C3H mice, 20–24 gm., received $10 \times 10^6$ 6C3HED ascites lymphosarcoma cells; female BDF1 mice, 18–22 gm., received $1 \times 10^5$ leukemia L1210 ascites cells or 0.2 ml. of a 15 per cent brei of Adenocarcinoma 755 subcutaneously in the axillary region.

Blood was obtained by opening the chest under ether anesthesia, cutting the vena cava, and pipetting the blood from the thoracic cavity into chilled centrifuge tubes. Serum and cells were separated by centrifugation and separately extracted with cold 0.2 M perchloric acid (PCA). Tissues were removed as rapidly as possible from the animals, homogenized in cold 0.4 M PCA, and extracted two more times with cold 0.2 M PCA. To avoid delay and possible enzymatic degradation of metabolites, blood, liver, and kidney samples were obtained from one set of animals, and intestine, spleen, and muscle samples from another group. Ascites tumor cells were removed from their hosts by capillary pipette after laparotomy, separated from the ascites fluid by centrifugation, and extracted 3 times with cold 0.2 M PCA. The solid tumors were homogenized in cold 0.2 M PCA after excision and extracted in the same manner. In every case the combined PCA extracts were neutralized with KOH, chilled, and the KClO₄ was removed.

These treated extracts were chromatographed either in a gradient elution system described previously (21) or in the manual system described below.

A combined nucleic acid hydrolysate was made in the following manner. The residue of tissues and tumors remaining after cold PCA extraction was suspended in 0.4 M PCA and heated for 50 minutes at 100°C. The insoluble protein residue was centrifuged, and the supernatant, containing the nucleic acid hydrolysate, was removed. The residue was washed once with 0.4 M PCA. The combined extracts were neutralized with KOH, chilled to precipitate KClO₄, and aliquots were plated for radioactivity measurements.³

To separate the nucleic acids the acid-insoluble fraction was made with 0.3 M in KOH and incubated, with occasional stirring, for 24–36 hours at room temperature. Cold 2.6 M PCA was added to make the solution 0.4 M in PCA. The precipitate, containing the DNA, was collected by centrifugation and washed 2 times with cold 0.4 M PCA. These washes were added to the supernatant fraction, which contained the hydrolyzed RNA. The DNA fraction was suspended in 0.4 M PCA, hydrolyzed by heating, and an aliquot was plated for radioactivity measurements. The RNA fraction was neutralized with KOH and an aliquot plated for radioactivity measurements.

**RESULTS**

**Blood levels.**—Normal mice were given intraperitoneal injections of 200 µg., 100 µg., or 50 µg. of APP-C¹⁴, and the radioactivity in the 0.2 M PCA extracts of the peritoneal fluid, serum, and blood cells measured 1, 2.5, 5, 15, and 30 minutes after drug administration. The determinations were done in quadruplicate. These data are presented in Chart 1, in which µg APP-C¹⁴/ml are plotted against time. (In both text and charts the radioactivity measurements have been converted to and are expressed as microgram-equivalents of APP. When chemical fractionation and characterization have not been made it must be understood that this refers to all forms of the drug present, not just to the free base. The concentration of the various metabolites of this drug, when reported, are also expressed as microgram-equivalents of APP and not as amount or concentration of each individual metabolite.) At all times studied, the APP concentration was greater in the peritoneal fluid than in

³ Leukemia L1210 and Adenocarcinoma 755 were obtained from Dr. Joseph Greenberg of this department.
Within 1 minute more than 90 per cent of the administered drug had been cleared from the peritoneal fluid, whereas less than 0.5 per cent remained 30 minutes after injection. APP also disappeared rapidly from the blood. The serum concentration was very low even 1 minute after injection of 200 μg of drug, had dropped fourfold in the next 30 minutes and was negligible 24 hours later. The 100-μg dose showed a similar decline, but the 50-μg dose was removed less rapidly from the blood. To compare blood levels following intravenous and intraperitoneal administration of APP, 200 μg was injected into normal mice by tail vein. It may be seen from Chart 1 that, although initial serum values were higher after intravenous injection, the results from these two routes were reasonably similar.

Incorporation of APP into the blood cells showed a different picture. The drug concentration was always lower at 1 minute than at 2.5 minutes, at which time the peak of uptake occurred (except for the 50-μg dose, in which the peak occurred at 5 minutes). Thereafter the APP concentration in the blood cells decreased, but much more slowly than in serum. At 24 hours there was a small but significant amount in the blood cells which was greater than that in serum. This behavior is somewhat similar to that observed for adenine, which had higher blood cell levels than serum levels (12). However, adenine was incorporated into blood cells more efficiently than was APP. The blood cell concentrations after intravenous administration of APP were initially higher than those for intraperitoneal injection, but declined more rapidly.

Chart 2.—Concentration of radioactivity in mouse tissues after intravenous injection of 200 μg of APP-C14.

Radioactivity was measured on neutralized perchloric acid extracts of tissues and whole blood. Values are averages of determinations on four animals.

Chart 3.—Total radioactivity in mouse tissues after intravenous injection of 200 μg of APP-C14. Recalculated from data in Chart 2.
Tissue distribution.—To determine the distribution of APP leaving the blood, 200 μg. of radioactive APP was injected intravenously into tumor-bearing mice, and the total radioactivity in the tissue extracts was determined at 1, 15, and 60 minutes. The results are shown in Charts 2 and 3, calculated in terms of concentration and total amount, respectively. At the times indicated, the chest was opened to remove the blood. Ascitic fluid was removed next, and then the solid tissues were removed, weighed, and homogenized in 0.2 M PCA. The extracts were each neutralized, and an aliquot was plated for measurements of radioactivity. The determinations were done in quadruplicate.

The highest concentrations at 1 minute were in blood, heart, liver, lung, and muscle; all these but liver decreased greatly in the next hour. The peak of APP concentration in the kidneys was at 15 minutes, presumably in relation to the high rate of urinary excretion of APP in the 1st hour (see below). Only in the tumor cells, liver, small intestine, and, to a lesser degree, spleen did the APP concentration increase during the first 2 hours after administration.

On the basis of the total amount of APP in the organs, however, liver, small intestine, and whole-body skeletal muscle (calculated on the basis of a sample of leg muscle) contained the greatest amounts of APP, followed by the tumor cells. This is remarkable in view of the fact that the total weight of liver and intestine were each two- to sixfold that of the tumor cells in each mouse. Brain, heart, lung, spleen, and kidney contained much smaller total amounts.

Urinary excretion.—Chart 4 shows the cumulative APP excretion in the urine after a single intraperitoneal dose of 200 μg. administered to normal and tumor-bearing mice, calculated as per cent of administered dose. Groups of five mice were used and their urines pooled. Normal mice excreted about 25 per cent of this dose in the 1st hour and another 25 per cent in the next 23 hours. Much less was excreted by the tumor-bearing mice, however; only 2.5 per cent was excreted in the 1st hour and a total of 33 per cent for the 24-hour period. The urinary metabolites were not characterized.

Separation and identification of metabolites.—Chart 5 represents a gradient elution chromatogram of liver extracts on Dowex-1-formate.

A perchloric acid extract of livers from two mice was made 1 hour after intraperitoneal injection of 200 μg. of APP-C14/mouse. The extract was neutralized, placed on a 10 X 100-mm. column of Dowex-1-formate, and eluted with a 300-ml. mixer and 5-ml. fractions collected. The solution in the reservoir was changed as indicated. The open trace is ultraviolet absorption at 280 mμ; the shaded areas represent APP radioactivity.

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mouse. The open tracing represents the ultraviolet absorption at 260 \text{m}_\text{A} and the shaded areas the radioactivity calculated as \( \mu \text{g} \text{APP/ml} \) in each fraction. The three most acidic APP metabolites appear in the leading edges of the adenosine mono-, di-, and triphosphate peaks, respectively. These compounds have been tentatively identified as the 5'- mono-, di-, and triphosphates of 1-ribosyl-APP, corresponding to the adenine nucleotides. Material from these fractions from a large number of separations was pooled to obtain sufficient material for analysis. The radioactive material in fractions 18–28 was separated from the bulk of the ultraviolet-absorbing material (adenyl acid) by paper chromatography in isopropanol-HCl (34) and isobutyric acid-ammonia (17) systems. The radioactive material was homogeneous in these systems. Acid hydrolysis liberated APP, as determined by paper chromatography and the ultraviolet spectrum. Analyses were made for ribose by the orcinol method and for phosphate (32). The ratio of APP:ribose:phosphate was 1:1.08:0.96. The values of \( R_F \) and absorption spectra corresponded with those reported by Way and Parks (33) for the APP-MP synthesized with purified enzymes.

The APP metabolites in fractions 72–84 and 105–110 have not been conclusively characterized, but they do release APP-MP upon mild acid hydrolysis. From this and their positions on the chromatograms it is tentatively assumed that they are the corresponding di- and triphosphates of 1-ribosyl-APP.

The first small peak on the chromatogram coincided with the location of free APP. To determine whether it also contained APP-riboside, this material was chromatographed on paper in the isopropanol-HCl system (34) and scanned for radioactivity with a Vanguard strip counter. A small peak containing about 10 per cent of the total activity was detected, in addition to the main fraction, which had the \( R_F \) and absorption spectrum of APP. The \( R_F \) of the minor component was not the same as any other metabolite studied. Amounts large enough for chemical analysis were not obtained, and it is only presumed, on the basis of expected chromatographic behavior, that this compound is APP-riboside.

The second peak, fractions 6–15, contained apparent APP degradation products. Paper chromatography of this fraction in isopropanol-HCl (34) and isomyl alcohol-disodium phosphate (4) revealed three major radioactive components and separated the products from extraneous ultraviolet absorption. These compounds have not been unequivocally identified. On the basis of \( R_F \), compared with those reported for APP urinary excretion products by Feigelson and Davidson (7), one of these compounds is tentatively identified as 4-hydroxypyrazolo(3,4-d)pyrimidine. The other two spots have strong end-absorptions in the ultraviolet region, but no maxima, and have not been identified. The orcinol test for ribose in these compounds was negative. They are presumed to be degradation products.

To facilitate the examination of a large number of tissue samples for APP metabolites, it was desirable to have a manual, stepwise chromatographic elution system, rather than the relatively slow gradient elution system. The following procedure was developed to separate the main APP metabolites into radio-pure fractions. (These fractions were of course not chemically pure.) Neutralized tissue extracts were poured onto 10 \( \times \) 40-mm. Dowex-1-formate columns. The pass-through plus a 10-ml. water wash contained the APP and presumptive APP-riboside. This is designated the "APP" fraction in subsequent charts and discussion. Fifteen ml. of 0.05 M formic acid eluted the degradation products; this is the "XPP" fraction; 15 ml. of 2.0 M formic acid removed the nucleoside monophosphate ("APP-MP"); 15 ml. of 4.0 M formic acid plus 0.2 M ammonium formate eluted the nucleoside diphosphate ("APP-DP"); and 30 ml. of 2.0 M formic acid plus 2.5 M ammonium formate removed the nucleoside triphosphate ("APP-TP"). These fractions were compared with those obtained on gradient elution chromatography and found to be identical. Each fraction was evaporated to dryness; the ammonium formate, if present, was sublimed off. Each sample was redissolved in 1 ml. of 0.05 N HC1 and an aliquot plated for radioactivity measurements in a micro-thin window gas-flow counter.

**Metabolism in normal tissues.**—To study the metabolism of APP in normal tissues of tumor-bearing mice, mice bearing 5-day Ehrlich ascites tumors each received an intraperitoneal injection of 200 \( \mu \text{g} \) of APP-C\(^{14}\), which is a therapeutic dose of this drug (10). Thirty, 60, and 120 minutes thereafter the tissues were removed, and the metabolites separated and measured as described above. All results are the averages of determinations on three to six mice analyzed separately. These results are given in Chart 6, where the concentration of all metabolites is expressed in terms of \( \mu \text{g} \text{APP/gm wet weight of tissue} \). In this and subsequent charts the three nucleotide forms are represented cumulatively.

In the liver of tumor-bearing mice the three nucleotides were the predominant forms of the drug, and the total amount of nucleotide increased...
during the 2-hour period studied. The exact significance of variations among the three different nucleotides is unknown but may just represent enzymatic action during removal of the liver (15). The free base content, while small, also increased slightly during this period, whereas the XPP fraction decreased slightly. It is of considerable interest to note that relatively large amounts of the di- and triphosphates were formed in this and other tissues. Thioguanine (21) and 6-mercaptopurine (26), on the contrary, are metabolized primarily to the monophosphate, with very small amounts of higher phosphates formed.

Smaller amounts of nucleotides were formed in the small intestine, but these still represented the most important metabolite on a quantitative basis. Again all three forms were represented. At 30 minutes considerable XPP was also found, but this rapidly decreased. The significance of the drop in nucleotide concentration at 60 minutes, followed by an increase at 2 hours, is uncertain.

Spleen contained a higher concentration of APP metabolites at 30 minutes than did intestine, but these decreased rapidly. Nucleotides were the predominant metabolites. The free base fraction was relatively more abundant in this tissue than in liver or intestine, and there were small amounts of degradation products. The profiles at 1 and 2 hours were approximately the same.

The kidneys contained relatively high amounts of free APP, especially at the first time period studied. The XPP fraction was also relatively large. Together they probably represent urinary excretion products. There was also considerable nucleotide formation, and it may be noticed that the diphosphate form predominated at the later periods.

Muscle contained the lowest concentration of the individual metabolites of the tissues studied. APP and XPP fractions were high compared with the total nucleotides, especially at the later periods.

In whole mouse blood nucleotide predominated at all times studied, but free APP was relatively high at the 30-minute point and decreased rapidly. Degradation products were prominent thereafter.

Metabolism in tumors.—To determine whether APP metabolism in tumors was qualitatively similar to that in normal tissues, extracts of 6-day Ehrlich ascites tumors from two animals were made 3 hours after the intraperitoneal injection of 200 µg. of APP-C14 per mouse and chromatographed in the same gradient elution system used above. The results are shown in Chart 7. The same kinds of metabolites were found as in the normal tissues, although in greater amounts. The first peak represents the free base, riboside, and degradation products. In the separation procedure used in the remainder of the experiments, these compounds have been better resolved.

To study the metabolism of APP in these seven tumors quantitatively, the same procedure was followed as with the normal tissues. Each tumor-bearing animal received an intraperitoneal injection of 200 µg. of radioactive APP, which is a therapeutic level of this drug (10). At the times indicated the tumors were removed, and the metabolites separated and measured as described above. All results are the averages of determinations of three to six mice analyzed separately.

The results of this investigation are shown in Chart 8. In comparison with normal tissues and other tumors, very large amounts of nucleotides were formed in the Ehrlich ascites carcinoma. Al-
though the monophosphate predominated at early time periods, the di- and triphosphates became relatively more important at later times. In all cases the nucleotides were the predominant metabolites, although free APP and the degradation products were also present. The concentration of nucleotides decreased to about 90 per cent of their original values in the 12 hours studied.

Considerably less nucleotides were formed in Leukemia L1210, but their amount increased during the 4-hour period studied. Much smaller amounts were present in the APP and XPP fraction.

Smaller amounts of metabolites were noted in Adenocarcinoma 755 than in the other two sensitive tumors, but this may be attributed at least in part to the fact that this tumor received the drug through the blood and did not receive an essentially intratumoral injection as did the ascites tumors. This makes the great increase in nucleotides over the 4-hour period even more remarkable, since blood levels of APP are very low even by 30 minutes after injection.

Sarcoma 180 ascites cells metabolized APP to form considerable amounts of nucleotides during the first 2 hours after administration of the drug, but much less was found thereafter, and by 12 hours relatively insignificant amounts remained.

In the thioguanine-resistant subline of Ehrlich ascites carcinoma (Ehrlich-TG-R), which is cross-resistant to APP, initial nucleotide formation was about one-half that formed in the sensitive parent cells. Although the rate of decline was not so rapid during the 2d hour as in the parent strain, the decrease in nucleotide concentration in the 3d hour was much more marked.

6C3HED lymphosarcoma cells formed less nucleotides than any other of the ascites tumors studied, and as time progressed the nucleotide concentration decreased.

Mecca lymphosarcoma cells metabolized APP to form relatively large amounts of nucleotides at the early time points, but this amount decreased rapidly. Considerable XPP was present at the first time period.

Test of APP incorporation into nucleic acids.—Three Swiss mice bearing 6-day Ehrlich ascites tumors were each given an intraperitoneal injection of 200 µg of APP-C¹⁴. After 3 hours the cells were removed, and the RNA and DNA hydrolysates obtained as described above. The RNA fraction contained 2.1 µg APP/gm wet weight of cells, while the DNA fraction contained 0.19 µg/gm, one-tenth as much. The alkaline hydrolysate of RNA was neutralized, the KClO₃ removed in the cold, and the preparation was poured onto a 10 x 40-mm. Dowex-1-formate column. The passage plus 10 ml of 0.01 M formic acid was collected, after which the column was eluted with 10 ml of 3 M formic acid. The first fraction would have contained any free APP or APP-riboside present and contained negligible radioactivity. The 3 M formic acid eluate, which would contain nucleoside monophosphates of APP, contained all

![Chart 7.—Gradient elution chromatogram of tumor extracts on Dowex-1-formate. A perchloric acid extract of Ehrlich ascites tumor cells from two mice was made 3 hours after intraperitoneal injection of 200 µg. of APP-C¹⁴ per mouse. The extract was neutralized, placed on a 10 X 100-mm. column of Dowex-1-formate, and eluted with a 300-ml. mixer, and 5-ml. fractions were collected. The solution in the reservoir was changed as indicated. The open trace is ultraviolet absorption at 260 mp; the shaded area represents APP radioactivity.

The radioactivity recovered, which was 96 per cent of that put on the column. These results support the conclusion that APP-C¹⁴ was incorporated into nucleic acids as nucleotide. The APP contained in the DNA fraction was not further characterized, although it seems probable that this was found in nucleotide linkage also. In the experiments reported below for other tumors and tissues it has been assumed that true incorporation was being observed. Neither separation of RNA and DNA nor characterization of the in-
corporated form of APP was performed in these cases.

Nucleic acid incorporation in normal tissues.—Swiss mice bearing 5-day Ehrlich ascites tumors were each given an intraperitoneal injection of 200 μg. of radioactive APP. Chart 9 presents the total incorporation of APP into the combined nucleic acids of several normal tissues, expressed as μg APP/gm wet weight of tissue. Small but significant amounts of incorporation occurred in all cases. Incorporation was progressive with time in liver, kidney, and spleen, but decreased in muscle. The incorporation into intestine was more than twice that of the other tissues, and all were much greater than that into whole blood, which presumably represents incorporation into leukocyte nucleic acids.

Nucleic acid incorporation in tumors.—The incorporation of APP into the combined nucleic acids of the tumors studied following a single intraperitoneal injection of 200 μg. of APP-C\(^{14}\) per mouse is shown in Chart 10. In all these tumors its radioactivity. The APP fraction contained free APP plus a small amount of APP-riboside; the XPP fraction contains the degradation products; APP-MP, -DP, and -TP are the mono-, di-, and triphosphates of 1-ribosyl-APP, respectively.

**Chart 8.**—Metabolism of APP in mouse tumors. 200 μg. of APP-C\(^{14}\) per mouse was injected intraperitoneally into tumor-bearing mice. Metabolites were separated on Dowex-1-formate by the manual chromatographic system described in the text, and the amount of APP in each fraction was calculated from its radioactivity. The APP fraction contained free APP plus a small amount of APP-riboside; the XPP fraction contains the degradation products; APP-MP, -DP, and -TP are the mono-, di-, and triphosphates of 1-ribosyl-APP, respectively.

**Chart 9.**—Incorporation of APP into the nucleic acids of normal tissues. Mice bearing 5-day Ehrlich ascites tumors each received an intraperitoneal injection of 200 μg. of APP-C\(^{14}\).
small but significant amounts of APP incorporation into nucleic acids occurred. There was, however, no correlation between the extent of this reaction and drug sensitivity or resistance. The drug-resistant tumors, Ehrlich-TG-R, Mecca lymphosarcoma, and Sarcoma 180, incorporated as much or more than did the sensitive tumors, Ehrlich and L1210. Adenocarcinoma 755 is a special case, since it was a solid tumor and had a slower growth rate than did the ascites tumors. It also did not receive an intratumoral injection, as did the ascites tumors. The low degree of incorporation in 6C3HED reflects the relatively low amounts of acid-soluble APP nucleotides in this tumor.

DISCUSSION

APP was converted to nucleotides in all normal and neoplastic tissues examined, and the combined nucleotide fraction was the predominant component at all times. In blood and muscle, however, the free base and degradation products were quantitatively more important than in the other tissues. It is most interesting that relatively equivalent amounts of mono-, di-, and triphosphorylated nucleosides were formed, although there was usually somewhat more of the monophosphate. Other purine antimetabolites that form nucleotides usually remain mostly at the monophosphate stage (21, 22). Unless evidence to the contrary is forthcoming, it seems probable that the nucleotides are the active forms of this drug.

For proper evaluation of these data, two matters must be considered. For effective carcinostatic activity, a drug must be present in a cell in its active form and at an adequate concentration over a sufficient length of time to affect its inhibitory action to such an extent that cell growth is significantly deterred. In addition, there must be some process or processes in the cell, essential to life or growth, that are inhibited, directly or indirectly, by this active form of the drug. In the case of APP the sensitive site(s) is not known. Therefore, it cannot yet be ascertained whether resistant tumors lack or easily bypass a system inhibited in sensitive tumors. Conclusions from the data presented in this paper as to grounds for drug metabolism may only be made with this important qualification in mind.

Certain features of APP metabolism are characteristic for sensitive and resistant tumors, considered as separate groups. While all the tumors studied formed nucleotides, in the sensitive tumors nucleotide synthesis increased with time (755, L1210) so that considerable concentrations were not only achieved but retained in the cells for some length of time. A second type of behavior in sensitive tumors is that such large amounts of nucleotides were formed initially that considerable amounts were present for a long time even though the concentrations decreased after the initial period of synthesis (Ehrlich).

In the resistant tumors either only small amounts of nucleotide were formed (6C3HED) or an initially high concentration of nucleotides declined rapidly so that what inhibition might occur was quickly released (Mecca, S-180, Ehrlich-TG-R). One recalls the finding of Bennett et al. (1) that sensitive and resistant tumors differed in a biochemical response to APP treatment only in the length of time affected.

The differences between the two groups appear to be quantitative, with only relatively small differences between inhibitory and sub-inhibitory concentrations of APP nucleotides. This suggests that frequent treatment might be more effective in the refractory cases. However, this would undoubtedly increase the already troublesome toxicity of this drug. These differences again support the conclusion that nucleotide(s) is the active agent in tumor inhibition. As pointed out above, an alternative explanation for the differences between the resistant and sensitive tumors could be the presence or absence of a sensitive site. In this case the quantitative differences in APP nucleotide concentrations noted above might have little significance for carcinostasis.

The toxicity of APP is expressed principally...
in the liver, which becomes fatty (28). This organ contained the highest concentration both of total APP and of APP nucleotides of the normal tissues studied. APP is the only purine analog which has such an effect. Bone marrow is also affected, although the intestine is not. It is interesting to note that intestine contained a relatively high concentration of drug metabolites, even though it is hardly affected by even very large amounts of drug (28). The liver toxicity would suggest that APP was active at the acid-soluble level, probably through some coenzyme function, rather than by interference with nucleic acid synthesis. Bone marrow cells are dependent on external sources for their purine supply (14), whereas intestine has a strong de novo synthesis process (11). In the latter case sufficient natural purine nucleotides might be present to protect APP-sensitive systems by competition; in marrow this may not be possible. On the other hand, intestine may not possess reactions sensitive to APP inhibition.

In connection with the toxicity of this drug, it is of interest to note that it is excreted much more slowly than other purine antimetabolites, such as 6-mercaptopurine, purine, 8-azaguanine, and thioguanine (5, 8, 19, 21).

These observations tend to suggest that the sensitive site is more concerned with some coenzyme functions at the acid-soluble level than with nucleic acid synthesis or growth. Biochemical studies previously reported in brief from this laboratory also suggest that APP acts at the acid-soluble level (13). Apparent stimulations of the incorporation of normal purines and purine precursors into nucleic acids were found at many of the times studied. These could most easily be explained by assuming that APP nucleotides caused disturbances in the sizes of the acid-soluble pools of purine nucleotides.

The incorporation of APP into the nucleic acids of normal and neoplastic tissues seems to be unrelated to its cytotoxic action. No correlation could be found between the rate or extent of APP incorporation and sensitivity to this drug. In the one case specifically examined, the incorporation into RNA was approximately tenfold that into DNA. In this connection it is interesting to note that APP has been found not to be a mutagen in Escherichia coli (91). These data also support the conclusion that APP acts at the acid-soluble level.

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The Metabolism of 4-Aminopyrazolo (3,4-d)pyrimidine in Normal and Neoplastic Tissues

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