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THE RENEWAL OF NUCLEOTIDES AND NUCLEIC ACIDS IN THE C_{57} MOUSE STUDIED WITH ADENINE-4, 6-C^{14}_{1}

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RENEWAL OF NUCLEOTIDES AND NUCLEIC ACIDS

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ABSTRACT

Adenine has been shown to be extensively utilized for the formation of acid-soluble adenine nucleotides and for the formation of RNA and DNA in the C_{57} mouse. The "apparent half lives" of the adenine in the nucleotides, RNA, and DNA of the organs of the mouse have been calculated, and a "fast" and a "slow"-component adenine were found in the adenine-containing fractions in several tissues. Evidence is presented that soluble adenine nucleotides serve as precursors for RNA and DNA, and that the equilibration of nucleotides with RNA is relatively rapid in the large and small intestines and probably also in the bone marrow. The desirability of more extensive data for nucleotide and nucleic acid specific activity as a function of time with several precursors including adenine, formate, glycine, 4-amino-5-imidazolecarboxamide, and phosphate is emphasized.

THE RENEWAL OF NUCLEOTIDES AND NUCLEIC ACIDS IN THE C_{57} MOUSE STUDIED WITH ADENINE-4, 6-C_1^{14}*

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INTRODUCTION

The rapid utilization of adenine-4, 6-C_1^{14} in the C_{57} mouse has been shown in an earlier study.[1] It was found that adenine was extensively and rapidly incorporated into derivatives of 5-adenylic acid and into nucleic acids. The results indicated that compounds present in the fraction soluble in cold trichloracetic acid (TCA) were possible precursors of the nucleic acids. Therefore, it seemed desirable to investigate the nature and interconversions of these compounds in more detail, particularly with reference to the equilibration of the adenine among the soluble nucleotides and the separated ribo-nucleic acid (RNA) and desoxyribonucleic acid (DNA).

The data presented in this and the following paper[2] confirm the previously reported extensive utilization of adenine for the formation of at least eight nucleotide derivatives in the mouse. The specific activities of the cold TCA-soluble 5-adenylic acid derivatives, RNA adenine, and DNA adenine in nine tissues of the mouse are compared from 2 hours to 16-29 days after administration of adenine-C^{14}. Apparent half lives of the 5-adenylic acid adenine, RNA adenine, and DNA adenine are calculated in the various tissues. Evidence is presented that RNA and DNA adenine may be derived from the soluble-nucleotide pool. The results show that the nucleic acids and the 5-adenylic acid in a given tissue are not in equilibration with those in other tissues.

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* A preliminary report of this work was presented at the American Chemical Society Meeting, September 6-11, 1953: Abstracts of Papers, page 5C.

METHODS

Administration of Adenine and Fractionation of Tissue

Adult male C_{57} mice, weight 28 to 30 g, age 6 to 7 months, were injected intraperitoneally with 1.3 mg of adenine-4, 6-C_1^{14}, ^{3} containing 2.2×10^7 dis/min, dissolved in 0.5 ml of 0.9% saline. The animals were allowed to feed ad libitum both before and during the course of the experiment on Purina laboratory chow. The mice were sacrificed by decapitation, and the organs were quickly removed and fractionated as described below. In the series of experiments reported here, the following organs have been studied: the skinned carcass (including bone),* liver, small intestine, large intestine (in Series II, these two organs were combined), stomach, kidney, lung, heart, and spleen and testis.

The carcass was homogenized in a Waring blender with cold 10% trichloroacetic acid (TCA) to extract the soluble nucleotides; a Potter homogenizer was used to homogenize the other tissue fractions. To ensure complete extraction of cold TCA-soluble material, each tissue was extracted 6 times with 10 volumes of cold 10% TCA, but generally only the first two extracts were saved. The TCA was removed by continuous liquid-liquid extraction with ether, and the remaining aqueous phase was subsequently analyzed as described below. The cold TCA-extracted tissue was washed twice with ethanol and twice with ether and then treated with 1 N KOH at 37° for 24 hours.[4] The RNA was obtained in the supernatant after acidification with 70% $HClO_4$. The DNA-protein-salt precipitate was washed with dilute $HClO_4$ and the washing was added to the main RNA fraction. Subsequently the precipitate was treated with KOH and $HClO_4$, as described above, two additional times in order to free the DNA completely of RNA contamination.**

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* The carcass fraction included the muscle and bone of the decapitated, eviscerated, skinned carcass; the feet and tail were discarded. It should be emphasized that the carcass fraction in this study consists of two very dissimilar fractions, the muscle and the bone marrow. Also, because of the small amount of tissue available, the spleen and testis were pooled. The intestines and stomach were rinsed with saline.

** X-irradiation experiments, to be reported in another communication, have indicated that two precipitations and washings of the DNA-protein are barely adequate to free the DNA from traces of contaminating RNA, whereas three precipitations remove all, or essentially all, the contaminating RNA from the DNA. This is extremely important in a tissue such as liver where the ratio of specific activity of RNA to DNA may be as high as 20 to 30.

The RNA extracts were neutralized with conc KOH, and the supernatants were used for subsequent analyses. Generally only the first KOH extract and washing were used, although the total activity present and uv absorption in the supernatants from the reprecipitations of the DNA-protein were determined as a check upon the washing procedure.

DNA was extracted from the DNA-protein precipitate with hot 10% TCA, and the TCA was removed from this extract by continuous liquid-liquid extraction with ether.

Determination of Specific Activity of Nucleotide and Nucleic Acid Adenine

Total 5-AMP. The specific activity of the soluble nucleotides hydrolyzable with $Ca(OH)_2$ to 5'-adenylic acid (5-AMP) in each fraction was obtained as previously described;[1] it is referred to as "total 5-AMP" or "acid-soluble 5'-adenylic acid." Generally, duplicate hydrolyses, chromatograms, and enzymatic analyses were made.

RNA and DNA Adenine. To concentrate the RNA adenine and to separate it from the salt present, two procedures were employed. In one procedure, the adenylic and guanylic acids were removed by 0.5 to 0.6 ml. of Dowex-1 resin from a suitable aliquot of the RNA solution made basic with 0.1 ml. of conc NH_4OH. The resin was washed once with water and the washings were discarded. The nucleotides were eluted with two 3-ml. portions of 0.2 N HCl, and the combined eluates were evaporated to dryness at 80° to 100° in an oven. Subsequently, the residue was dissolved in 4% acetic acid and chromatographed on Whatman No. 1 filter paper as previously described.[1] In the other procedure, the nucleotides were hydrolyzed to the free purines by heating a suitable aliquot of the RNA fraction at 100° for 1 hr in 1 N HCl. Subsequently the purines were adsorbed on 0.2 ml of Darco G-60 charcoal and then eluted with two 4-ml portions of 73% ethanol–24% water–3% conc NH_4OH. The combined eluates were evaporated to dryness and the residue was dissolved in a small amount of 4% acetic acid and chromatographed as described above. The adenine was eluted and the specific activity was determined with xanthine oxidase.[1] The two methods gave similar results; the second method is preferable, however, as less extraneous material was found on the chromatograms.

DNA adenine was adsorbed on Darco G-60 charcoal after acidification with dilute acetic acid. Subsequently the procedure was identical to that described for RNA adenine.

Duplicate determinations were usually made for both RNA and DNA adenine, and the specific activities determined generally agreed within 5 to 10% except for tissues or time intervals where the specific activity was low (10 dis/min/μg adenine), in which case duplicate values occasionally differed by as much as 25%.

Counting Procedures.

Direct plating techniques were used for the fractions described. Duplicate samples and (or) duplicate counts were made of all samples. Suitable self-absorption corrections were applied to the cold TCA, RNA, and DNA fractions. For the RNA and DNA samples before chromatography, the corrections were large (20 to 50%), therefore values for the total amount of radioactivity incorporated are approximate. After chromatography, no self-absorption corrections were necessary.

The radioactivity measurements were made with a Tracerlab SC-16 windowless flow type counter using platinum dishes approximately 3.7 cm^2 in area. The counting efficiency was approximately 55%, and the background was equivalent to 35 dis/min.

RESULTS

Two series of mice were used for the experiments described in this paper. Series I was comprised of 8 mice, sacrificed at 2 and 6 hours, 1, 2, 3, 7, 16, and 29 days after adenine-4, 6-C_1^{14} was administered. Mice in Series II were sacrificed at 2 hours, 1, 3, 7, and 16 days. The data are reported in detail for the first series only, inasmuch as the results of the two series of experiments are in general agreement.

The results are summarized in Table I, in which are recorded the specific activities of the acid-soluble 5-adenylic acid, the RNA adenine, and the DNA adenine. The percentage of the administered adenine incorporated into each of these fractions in the individual tissues at the time of maximal incorporation is summarized. In addition, the "apparent half life" of the adenine in these fractions is given for the tissues studied. As shown in Fig. 1 for the small intestine, values for this half life of the adenine in the total 5-AMP, RNA, and DNA have been obtained by plotting the specific activity of the adenine of a tissue fraction as a function of time on semilogarithmic graph paper and determining the time required for the specific activity to decrease by 50%. Generally two component rates could be obtained. The slower rate component was calculated

SPECIFIC ACTIVITY AND HALF LIFE OF RNA AND DNA ADENINE

AND 5-ADENYLIC ACID IN VARIOUS TISSUES

(dis/min/μg Adenine)

Time after Injection	Small Intestine			Large Intestine		
	5-Adenylic Acid	RNA Adenine	DNA Adenine	5-Adenylic Acid	RNA Adenine	DNA Adenine
2 hours	2690	424	109	2120	475	112
6 "	2280	662	254	1970	697	240
24 "	1355	883	465	1200	950	342
48 "	472	495	343	710	650	455
72 "	278	256	176	376	360	225
7 days	81	79	29	108	97	48
16 "	22	31	12	33	28	14
29 "	4.6	6.5	(7.2)*	—	—	—
Half life	18 hr	17 hr	21 hr	23 hr	19 hr	18 hr
(Series I)	5.6 days	6.0 days	7.0 days	5.5 days	5.0 days	5.0 days
% "Fast components"	94%	93%	89%	89%	83%	79%
% Incorp. at max.	7.9%	3.6%	1.8%	3.1%	1.14%	0.53%
(Series II) **	23 hr	19 hr	24 hr			
	4.0 days	5.8 days	7.7 days			
% "Fast Component"	92%	82%	86%			

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TABLE I, Cont.

Time after Injection	Carcass			Liver		
	5-Adenylic Acid	RNA Adenine	DNA Adenine	5-Adenylic Acid	RNA Adenine	DNA Adenine
2 hours	278	160	109	1600	133	5.0
6 "	221	204	196	1540	225	10
24 "	205	229	162	990	385	18
48 "	200	210	116	585	306 ?	15
72 "	179	193	81	445	387	18
7 days	150	151	24	215	330	15
16 "	87	89	6.5	75	105	11
29 "	(83) * ?	(65) * ?	(7.2) *	11.4	26	(10.9) *
Half life	-	-	35 hr	17 hr	-	-
(Series I)	12 days	12 days	7.0 days	5.2 days	5.5 days	16 days ?
% "Fast component"	-	-	82%	71%	-	-
% Incorp. at max.	11%	1.3%	0.9%	8.8%	1.4%	0.03%
(Series II)	22 hr	-	27 hr	18 hr	-	-
	12.7 days	15.3 days	7.3 days	6 days	9 days	-
% "Fast component"	65%	-	82%	67%	-	-

TABLE I, Cont.

Time after Injection	Lungs			Kidneys		
	5-Adenylic Acid	RNA Adenine	DNA Adenine	5-Adenylic Acid	RNA Adenine	DNA Adenine
2 hours	1400	259	17	1460	172	1.6
6 "	1295	306	15	1860	332	4.2
24 "	1075	450	48	1230	549	15.5
48 "	660	530	46	705	540	36.3
72 "	440	400	36	525	512	41
7 days	195	305	15	208	343	30
16 "	78	166	11	53	104	24
29 "	-	-	-	21	35	21
Half life	24 hr	-	~ 32 hr	22 hr	-	-
(Series I)	7 days	10.3 days	~20 days	4.8 days	5.5-7.0days	~40 days
% "Fast Component"	80%	-	-	75%	-	-
% Incorp. at max.	0.62%	0.11%	0.01 %	3%	0.44%	0.03%

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TABLE I, Cont.

Time after Injection	Stomach			Spleen and Testes		
	5-Adenylic Acid	RNA Adenine	DNA Adenine	5-Adenylic Acid	RNA Adenine	DNA Adenine
2 hours	1890	287	36	865	64	13
6 "	1660	530	93	765	80	33
24 "	965	676	177	645	123	31
48 "	635	880	272	455	147	37
72 "	470	438	143	393	135	29
7 days	215	153	52	186	128	33
16 "	82	67	21	76	72	25
Half life	18-24 hr	15 hr	16 hr	28 hr	-	-
(Series I)	6.5 days	7.5 days	6.8 days	7.7 days	11-14 days	-
% "Fast component"	70-80%	~60%	68%	70%	-	-
% Incorp. at max.	0.75%	0.25%	0.09%	1.4%	0.48%	0.06%
(Series II)	12-17 hr	-	-	26 hr	-	-
	8 days	-	~10 days	11.5 days	14-19 days	-
% "Fast Component"	83-87%	-	-	71%	-	-

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TABLE I, Cont.

Time after Injection	Heart		
	5-Adenylic Acid	RNA Adenine	DNA Adenine
2 hours	257 ?	-	2.5
6 "	220	-	8
24 "	206	-	8
48 "	195	-	5
72 "	176	-	12
7 days	117 ?	-	7
Half life (Series I)	~11 days	-	-
% Incorp. at max.	0.14%	0.01 %	< 0.01%

* Values in parentheses not used to calculate half life.

** Large and small intestine.

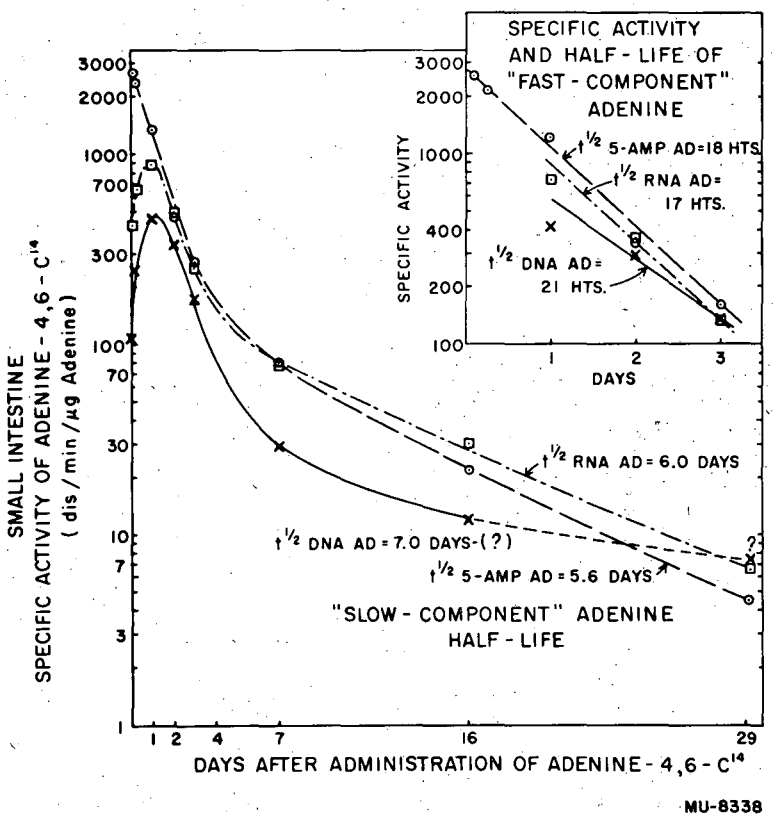

Fig. 1. Specific activity and half life of 5'-adenylic acid, RNA, and DNA adenine of small intestine of mice from 2 hours to 29 days after administration of adenine-C^{14}. Specific activity and half life of "fast component" adenine shown in inset.

from the data obtained from 7 to 16 days or 7 to 29 days after the injection of the adenine. This curve was graphically extrapolated to zero time, and the specific activities of the slow component were subtracted from the total specific activities found at the early time intervals. The resulting values were plotted and a graphical representation of the rapidly metabolized component was obtained. This is illustrated in the inset of Fig. 1. In addition, the approximate percentage of the activity incorporated into the "slow" and "rapid" components has been calculated from the "time = 0" values of the component curves of the total 5-AMP, and from the ratio of the specific activity of the "slow" curve to the total specific activity at the time of maximum specific activity for the RNA and DNA. These values are also summarized in Table I for both series of animals.

DISCUSSION

The incorporation of phosphate, glycine, formate, 4-amino-5-imidazole carboxamide, orotic acid, adenine, and other precursors into nucleic acids of rats and mice has been studied by numerous investigators in recent years. It has been shown that these compounds can be utilized for the formation of nucleic acids, but data for the distribution of the administered precursor in the separate tissues and in the various compounds into which they are incorporated are limited. With a few exceptions, kinetic studies have not been made, and even these few kinetic studies have usually been confined to one tissue or to only several time intervals.

The earliest studies of nucleic acid renewal were those of Hevesy and co-workers,[5] who studied the incorporation of P^{32}-phosphate into the DNA of numerous tissues of the rat 4 days after a single administration of the radioactive precursor. From the data obtained, a minimum daily renewal rate for the DNA in numerous tissues was calculated. This rate ranged from an apparent daily renewal rate for DNA of 15% in the mucosa of the small intestine to 0.6% in kidney and brain. A second series of experiments compared the incorporation of P^{32}-phosphate in the RNA to DNA of several tissues two hours after a single administration. The RNA/DNA specific activity ratio ranged from 33 for liver to 2 for intestine.[6]

These early studies set the pattern for many that have followed using labeled precursors of nucleic acids. In these investigations, the emphasis has been upon the degree of incorporation of nucleic acid precursors at

usually one to three time intervals, ordinarily short, after single or multiple administration of the precursor, or upon the relative utilization of the precursor for RNA as compared to DNA formation. In many experiments pooled tissues or pooled nucleic acids have been investigated, and the incorporation of the precursor into the low-molecular-weight nucleotides has been essentially neglected.

The most extensive kinetic data have been obtained with P^{32}-phosphate, particularly in respect to relative incorporation into RNA in the cellular fractions of liver,[7, 8, 9, 10, 11] and carcinoma.[11, 12] These investigations have shown that the nuclear RNA incorporates P^{32}-phosphate more rapidly than the other cellular fractions. Similar results have been obtained with glycine,[11, 13] formate,[13, 14] orotic acid,[15, 16, 17] and adenine.[1, 18, 19]

Experiments from which an "apparent rate of renewal" of nucleic acids can be calculated are limited. From a detailed analysis of P^{32} specific activity in the nucleic acid of cellular fractions of mouse carcinoma, Barnum and co-workers[12] have concluded that the renewal of phosphate in the RNA fraction was rapid, from 38% to 150%/hr. It was also calculated that the phosphorus renewal of DNA was 5%/hr, or twice the net increase of DNA. In rats, the rate of uptake of P^{32} into DNA of liver, intestine,[20] and lung[21] has been compared with the mitotic rate. In this study, the whole acid-soluble phosphorus was considered to be the immediate precursor, and specific activities were determined at numerous time intervals up to 72 hours. When compared to daily mitotic rates of 0.7%, 53%, and 4.1%, respectively, the uptake of P^{32} was found to be approximately twice the rate of cell formation, or 1.24%, 115%, and 10.7%.

In experiments with P^{32}-phosphate, estimations of renewal rate of nucleic acids have been based upon the rate of uptake of phosphate and upon the assumption that nucleic acid phosphorus is derived from the free phosphate,[5] an unknown "x" which equilibrates rapidly with the inorganic phosphate,[12] or the acid-soluble phosphate.[20, 21] This is possible because of the relatively rapid equilibration of phosphate between inorganic phosphate, total acid-soluble phosphate, and acid-soluble organic phosphate, and also because these fractions are normal constituents of animal tissue; thus the changes in specific activity are not too rapid and can be determined. Therefore, rather similar conclusions are reached regardless of which one of these classes of compounds is assumed to contain the immediate precursor of the nucleic acids.

When precursors of nucleic acids such as adenine, orotic acid, or glycine are administered, renewal rates based upon the rate of incorporation of the precursor have several limitations. In the first place, assumptions must be made concerning the route by which the precursor is incorporated. Although some adenine may enter RNA directly, orotic acid and glycine must go through intermediates. The specific activity of these intermediates must be known in order to calculate more than the relative renewal rates. Even this calculation is probably misleading, as the specific activity of the precursor varies from tissue to tissue, especially as a function of time. In addition, a short time after administration of orotic acid or adenine, they are converted into other compounds, primarily uridylic[15, 16] or adenylic acid derivatives,[1, 2] so little or no free orotic acid or adenine is present. Renewal rates based upon uptake must necessarily assume that the exclusive source of the "building blocks" during the time interval studied is the labeled precursor, a situation not likely to be realized with either adenine or orotic acid for longer than several hours after administration.[1, 2, 15] Thus, experiments in which adenine is administered over a several-day interval, and in which the ratio of specific activity found in the nucleic acid adenine to that administered is reported only for a single time after administration, can give a mimimal value for nucleic acid "turnover."[22, 23] Long-term experiments with adenine[22, 23] and formate[24] in which the incorporation has been determined at only two time intervals after administration, although permitting a comparison of "apparent retention" to be made in the tissues studied, do not yield sufficient data to calculate a valid "half life." In such experiments, it has been assumed that the maximal specific activities are obtained within 1 day after administration of the precursor was concluded, an assumption which may often be unjustified.

From the specific activities of the adenine and guanine in the liver RNA and DNA determined 4, 7, and 12 days after glycine-C^{14} was administered to rats, a half time of about 220 hours was obtained for the adenine and guanine of RNA and 150 to 160 hours for that of the DNA. The free glycine was found to have a half life of 9 and 200 hours.[25]

In experiments that have been reported with orotic acid, the complex nature of the specific activity–time relationships in the various cellular fractions of rat liver has been shown. A half life of approximately 14 hours can be calculated for the nuclear RNA from the 2 to 16-hour data,[16] and a

half life of the order of 5 to 7 days for the cytoplasmic RNA based on 20- to 91-hour data.[15]

In relatively short-term experiments with adenine-C^{14}, Fresco and Marshak have calculated apparent half-time values of 12 and 24 hours, respectively, for the nuclear RNA and cytoplasmic RNA of mice.[18] The importance of a knowledge of the full course of incorporation and disappearance of precursor and the necessity of as complete kinetic data as possible in order to estimate renewal rates was stressed. It was realized that other less rapidly metabolized components may have been present.

In our previous investigation[1] the utilization of adenine-4, 6-C^{14} for the formation of 5-adenylic acid derivatives and nucleic acids of various tissues of C_{57} mice was studied. It was shown that the adenine was extensively incorporated into the nucleotides as well as the nucleic acids of the mouse. Preliminary values were obtained for the renewal rate or apparent half life of the adenine in the nucleotides and pooled nucleic acids. The desirability of extending this investigation to more numerous time intervals, to more tissues, and to the separated nucleic acids was apparent. In the investigation here described the specific activities of the soluble 5'-adenylic acid derivatives ("total 5-AMP"), RNA adenine, and DNA adenine have been determined in 4 tissues of the mouse up to 29 days after the single administration of adenine-4, 6-C^{14} and in 5 other tissues up to 16 days.

The data presented in Table I show that the adenine is rapidly incorporated and renewed in the cold TCA-soluble 5'-adenylic acid derivatives of the mouse. The more numerous time intervals that have been studied in this investigation have made it possible to estimate that 70 to 94% of the adenine incorporated into the total 5-AMP of all the tissues with the exception of the carcass and heart had an apparent half life ranging from 18 hours to 28 hours. In addition, a small amount of the total 5-AMP in these tissues was renewed at a relatively slow rate with a half life of 5 to 7 days. A comparison of the specific activity in the several tissues at two hours to the half life shows, as might be expected, that the tissues with the shortest half life for the 5-AMP also incorporate the most adenine. A comparison of the specific activity of the total 5-AMP in the various tissues at numerous time intervals indicates that the 5-AMP in one tissue is not rapidly equilibrated with that in another.

The present knowledge of the mechanism of nucleotide synthesis suggests

that free adenine is not on the normal route of nucleotide biosynthesis from small-molecular-weight precursors such as glycine and formate. However, the results of this and other investigations show that adenine is rapidly incorporated into soluble nucleotides when it is administered to a rat[26] or a mouse.[1, 2] Several mechanisms may be suggested for the incorporation of adenine into the adenylic acid nucleotides. Saffran and Scarano[27] have shown that 5'-adenylic acid is formed when adenine, ribose-5-phosphate, and ATP are incubated with a pigeon liver extract. It has been shown that 5'-phosphoribosylpyrophosphate in the presence of an enzyme isolated from yeast can condense with adenine to form adenylic acid.[28] These mechanisms yield a net synthesis of adenylic acid. Another possible mechanism would be by a trans-N-glycosidase type of reaction which has been shown to occur with desoxyribosides, but not as yet with the ribosides or ribotides.[29, 30] This mechanism would not result in a net synthesis of nucleotide, and could not account for the rapid disappearance of the free administered adenine. It would lead, however, to a rapid incorporation of radioactive adenine into soluble nucleotides.

A half life of 18 hours found for the total 5-AMP of the small intestine is equivalent to approximately 4% renewal/hour. The observed renewal based upon the uptake of adenine-4, 6-C^{14} in 2 hours was 16% or twice the expected renewal based upon its subsequent disappearance from the 5'adenylic acid pool. The renewal based upon uptake is a minimum renewal rate as this calculation assumes that free adenine is present in excess throughout the entire 2 hours, and that no renewal of 5'-adenylic acid occurs from other sources during this period. It is also assumed that no labeled 5-AMP leaves the pool during this time interval. It is known that considerable free adenine is present 1/2 hour after administration, but only a very small amount is present at 2 hours.[2] Using the 1/2 hour total 5-AMP data,[2] one can calculate a renewal rate based on the uptake of adenine of approximately 20%/hour. Although the amount of adenine injected in these experiments is small compared to the amount of adenine derivatives in a mouse, it is not a true tracer dose, as little or no free adenine is normally present. The initial reaction incorporating the adenine into nucleotides and perhaps nucleic acids may be dependent on the amount of adenine present, and even the small amount administered may cause an increased rate of formation of 5'-adenylic acid nucleotides. By chromatographic methods,[2] greater

quantities of inosinic acid were observed 1/2 hour after administration of adenine than 24 hours later, which probably represents enhanced deamination of 5-AMP to restore the tissue levels to normal. In part, the direct incorporation of adenine may serve merely as a detoxification mechanism for the animal to eliminate adenine by pathways other than by direct oxidation to 2, 8-dioxyadenine. This substance is found when adenine is administered in larger amounts than used in these experiments, and can cause kidney damage.[31] Conversion of adenine to 2, 8-dioxyadenine probably becomes an important metabolic pathway when the quantity of adenine is so large that sufficient ribose acceptor is not readily available to convert adenine into adenylic acid. The inhibition of formate[14, 32] and glycine[33] utilization for nucleic acid formation may be due to the preferential formation of nucleotides from adenine.

In addition to being rapidly incorporated into nucleotides, adenine is also incorporated into RNA and into DNA. The maximal specific activity and time of maximal specific activity in the RNA and DNA are different for the various tissues studied. The decreasing sequence of specific activity in the RNA at 1 day was large intestine, small intestine, stomach, kidney, lung, liver, carcass, and spleen and testis. Brown et al. found the sequence in the rat to be liver, kidney, intestine, spleen, and testis,[22, 34] whereas Abrams found, in agreement with our experiments, that the specific activity of the intestine was higher than that of the liver.[33] Although the experiments are not strictly comparable, the cause of the differences observed is not apparent. The different specific activity–time relationships found for the soluble nucleotides, RNA, and DNA, would indicate that very little exchange occurs between the tissues. A similar conclusion has been reached from relatively short-term experiments with orotic acid in rats.[15, 16]

In the small intestine, large intestine, and stomach, two rate components for the disappearance of the adenine-C^{14} from the RNA are evident, a rapid-half-life component of 15 to 19 hours, and a slow component with a half life of 5 to 7.5 days. In the intestines, the short-half-life fractions comprise 83% to 93% of the radioactivity incorporated into the RNA. In the liver, sufficient data were not obtained at short time intervals to obtain a fast component comparable to that reported by Fresco and Marshak.[18] A half life of 5.5 days was found for the liver in Series I based upon the 3- to 29-day data. The adenine of the RNA of other tissues had apparent

half lives ranging from 5.5 to 7.0 days for the kidney to 10 to 15 days for lung, carcass, and spleen and testis.

Calculations of apparent half life of RNA fractions based upon the data for apparent retention values of adenine derived from formate-C^{14} [24] yield similar apparent half lives for liver (7 days), kidney (7.7 days), testis (11 days), spleen (5.7 days), but since the calculations are based solely on 1- and 24-day values, a significantly different value of 4 days is obtained for the small intestine where two component rates appear to exist. It is stated[24] that the half life of the DNA and RNA in the small intestine can be estimated to be about 1 to 2 days, but the source of this value is not apparent.

Four tissues (small intestine, large intestine, stomach, and carcass) incorporated appreciable amounts of adenine into the DNA fraction. In the first three tissues, a fast-component adenine with a half life of 16 to 20 hours was found, while the carcass had a fast-component adenine with an apparent half life of 35 hours. In addition, each of these tissues had a slow-component adenine with a half life of 5 to 7 days. Seventy to ninety percent of the radioactivity was incorporated into adenine of the fast DNA component. An apparent half life of 18 to 23 hours for the total 5-AMP, RNA adenine, and DNA adenine is to be compared with the estimated life for cells in the intestinal mucosa in the rat of 40 hours.[20] This may be evidence that little reutilization of these fractions occurs, but the actual physical loss of cells from these tissues should be borne in mind. The other tissues incorporated only a small amount of adenine in the DNA, and the estimates of half life ranging from 16 to 40 days have a high degree of uncertainty.

It should be reemphasized that the half lives determined by the techniques used in this series of experiments are only apparent half lives, and represent only the time required for one-half of the labeled adenine molecules which have been incorporated into a fraction to be replaced by unlabeled adenine molecules, either synthesized de novo or obtained from an exogenous purine source. The molecular fractions whose half lives are determined in this study are only those fractions which have become labeled during the course of the experiment. Fractions such as the DNA of the liver and heart, which are being renewed only very slowly, take up very small amounts of the adenine initially, so that a satisfactory estimate of renewal cannot be obtained. In other tissues, such as intestine, there may also be fractions

of RNA or DNA that are not being renewed, and which therefore do not become labeled and whose half life is not evident. As will be apparent from the following discussion, any such fraction in the intestine is probably small. In this study, no attempt has been made to fractionate either the DNA or RNA, either on the basis of physical properties or on the basis of cellular fractions. Some evidence has been obtained for the inhomogeneity of DNA after labeling with formate[24] and considerable evidence has been obtained, particularly in liver and tumors, for the cellular inhomogeneity of RNA. Evidence has also been obtained for the inhomogeneity of the RNA of a given cellular fraction at short time intervals after administration of P^{32}-phosphate, glycine-C^{14}, and orotic acid-C^{14}.[35] The two-component rate curves obtained for the disappearance of adenine from the nucleotide and nucleic acid fractions of several tissues clearly indicate the not unexpected over-all inhomogeneity in metabolic rates of a tissue.

It is evident from an examination of the data in Table I that the RNA/DNA-adenine-C^{14} ratio varies widely from tissue to tissue, and it also varies as a function of time, so that a comparison of the ratios at a single time interval may be misleading and may have only limited validity. In part, the change of this ratio with time may account for the diverse RNA/DNA specific activity ratios found in the literature after the administration of a given precursor.

As has been previously noted,[1] the maximal specific activity of adenine in the nucleic acids in all tissues except carcass-DNA, occurs 24 to 48 hours after the adenine has been administered. It has been shown[1, 2] that adenine is essentially completely converted to nucleotides or to the catabolites of adenine, principally hypoxanthine and allantoin, within two hours after administration. The catabolites of adenine have been shown to be poor precursors of nucleic acids,[36] and thus it would appear that the 5-adenylic acid nucleotides are serving as precursors of the adenine in nucleic acids. However, if it is assumed that at short time intervals the adenine is first incorporated into the nucleotides before being incorporated into the RNA, and that the mean specific activity of the 5-adenylic acid derivatives is 2000 dis/min/μg adenine for the first 2 hours in the intestines, a renewal of over 20% of the RNA in the intestine would be required to obtain the observed specific activity of 425 to 475 dis/min/μg adenine in the RNA of the small and large

intestine.* In other words, an equilibration exists between the soluble nucleotides and RNA that is much more rapid than the apparent renewal of the RNA. This would be in qualitative accord with the results of experiments with P^{32}-phosphate by Barnum et al.[12] The other alternative is a "direct" incorporation of adenine into RNA, or a route of incorporation that does not go through the 5'-adenylic acid pool. Experiments in which the RNA of cellular fractions of the intestine were isolated after adenine administration would be of considerable interest.

If similar calculations are made for the rate of incorporation of adenine into the DNA of the intestines during the first two hours after administration of adenine-C^{14}, it is found that the calculated renewal of 7% to 8% based upon the observed half life of adenine in the DNA of the intestines is of the same order of magnitude as the incorporation of adenine-C^{14} if it is assumed that compounds of the 5'-adenylic acid pool serve as intermediates. The apparent lag in incorporation of adenine-C^{14} into DNA as compared to RNA[2] is consistent with a mechanism of relatively rapid reversible formation of RNA from the soluble nucleotides and the slower formation of DNA also from nucleotides.

Additional evidence that the 5'-adenylic acid nucleotides are direct precursors of RNA adenine, perhaps sole precursors in the intestines, is the striking correspondence of specific activity of the RNA adenine and 5-AMP adenine in both the small and large intestine subsequent to two days after administration. A close correspondence of specific activity of 5-AMP adenine and RNA adenine of the carcass was also found, but until these values have been separately determined in muscle and bone marrow the significance is uncertain. In most of the tissues studied, a close relationship

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* It has been assumed that the 5-AMP nucleotides have uniform specific activity within the cellular fractions. Isolation of the cold-TCA-soluble fractions in the cellular fractions by centrifugation in 0.25 M sucrose indicated that 90% of the radioactivity in the soluble nucleotides was localized in the cytoplasmic supernatant fraction and 10% in the mitochondrial fraction, and the nuclear and microsomal fractions contained only 1 to 2% of the radioactivity.[1] A similar distribution of the uridylic acid nucleotides is suggested from experiments with orotic acid,[16] except that the microsomal rather than the mitochondrial fraction was found to contain approximately 7% of the acid-soluble radioactivity at 2, 4, and 8 hours. The experiments of Naora and Takeda[37] in which the cellular fractions were isolated in nonaqueous media suggest that a large fraction of the ATP of a cell may be localized in the nucleus and in the mitochondria. If this is the case, a study of the cellular distribution of nucleotide specific activity as a function of time is desirable.

was found between the half life of 5'-adenylic acid adenine and RNA adenine. One exception was the spleen and testis fraction, which is composed of two separate tissues.

The liver, a tissue in which the most extensive studies of specific activities of nucleotides and nucleic acid fractions have been made,[11, 16, 38] does not appear to have the close correspondence of nucleotide and RNA specific activities after adenine-4, 6-C^{14} administration. At periods up to 3 days after adenine administration the specific activity of the RNA adenine is less than that of the 5-AMP adenine, while at later periods it is greater.

Considerable indirect evidence has been accumulated in the past several years that soluble nucleotides within a tissue may serve as precursors of nucleic acids.[1, 15, 16, 38, 39, 40] The present concept of RNA structure as a polynucleotide chain in which the individual nucleotides are linked by phosphodiester linkages between the 3'- and 5'-positions of the ribose moieties[41, 42] focuses attention on the 5'-nucleotides as logical intermediates for the formation of RNA and perhaps even DNA.* The 5'-nucleotides have been found to be normal constituents of tissues.[39, 43]

Marrian has presented evidence that regenerating rat liver is able to utilize purines, probably acid-soluble, from its environment for the formation of DNA.[40] Unfortunately, not sufficient data of the amount of liver removed are given to enable one to calculate if the newly formed RNA could have also arisen in part from the soluble nucleotide fraction.

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* If the 5'-nucleotides are precursors of nucleic acids, the interpretation of experiments in which the specific activities of $P^{32}O_4^{---}$ in individual nucleotides of RNA after alkaline hydrolysis have been determined is open to question, as the nucleotide isolated would contain the P^{32} which was initially present on the adjacent 5'-nucleotide at the time of synthesis of the nucleic acid. Moldave and Heidelberger[35] have, however, compared the specific activities of nucleotides obtained after hydrolysis of microsomal RNA by ribonuclease (2'- or 3'-nucleotides) and snake venom diesterase (5'-nucleotides). No marked difference, as might have been expected, in the specific activity of the 2'- and 3'-nucleotides and the 5'-nucleotides of the same base was found. By both methods of hydrolysis of RNA obtained from the microsomal fraction 40 minutes after administration of the $P^{32}O_4^{---}$, the adenylic and the uridylic acids had the highest specific activities. Clearly, much more information concerning the synthesis and degradation, as well as structure, of nucleic acids is necessary before such results can be unequivocally interpreted.

The experiments with orotic acid-C^{14} reported by Huribert and Potter[15, 16] show that it was preferentially and rapidly utilized in the liver of rats. At short time intervals after administration, the orotic acid was converted primarily into acid-soluble derivatives of uridylic acid. Subsequently, these acid-soluble derivatives of uridylic acid were converted in large measure into RNA in the liver. From 2 to 16 hours after administration of the orotic acid, the specific activity of the nuclear RNA maintained a specific activity of about one-half of that of the acid-soluble uridylic acid derivatives.* Up to 16 hours, the cytoplasmic RNA specific activity did not parallel that of the acid-soluble uridine phosphates; the data enabling a direct comparison to be made at later time intervals were not given. It would be expected, however, that a closer correlation between RNA specific activity and that of the soluble uridylic acid derivatives would be found during the 16- to 91-hour period in liver. Fresco and Marshak[18] have compared the acid-soluble adenine, nuclear RNA adenine, and cytoplasmic RNA adenine in mouse livers and have obtained rather similar results.

Schmitz et al.[38] have compared the specific activities of the nucleotides obtained from RNA and the acid-soluble nucleotides in Flexner-Jobling rat carcinoma after administration of glucose-1-C^{14} and have found a close correlation at periods varying from 8 to 18 hours after administration. The nucleotides derived from DNA had consistently lower specific activities than those derived from RNA, and the results are not incompatible with the formation of desoxyribotides from ribotides. From experiments with cytidine in the rat, Rose and Schweigert have also found evidence for DNA formation from nucleotides.[44]

In order to further test the suggestion that the acid-soluble nucleotides serve as a pool of nucleic acid precursors it would be extremely desirable to have additional experiments in which a comparison of the specific activity of the acid-soluble nucleotides and nucleic acids is made in several tissues and at several time intervals, particularly after the administration of formate or glycine. LeBage has compared the incorporation of glycine-2-C^{14} into acid-soluble guanine and adenine derivatives and nucleic acid guanine and adenine in in vitro systems using cell suspensions and homogenates of mouse liver, Ehrlich carcinoma, and Gardner lymphosarcoma.[45] In all

* See Footnote, page 22.

cases the specific activity of the nucleic acid fractions were less than those of the soluble nucleotides. A preliminary report of a similar comparison in vitro has appeared.[46] The data from studies that have compared the specific activity of the ATP of the muscle with that of the PNA and DNA of the viscera after formate or glycine[47] or adenine[48] administration are not applicable.

It has been shown[11, 14] that adenine and phosphate are very similar in the extent to which they are utilized for RNA and DNA formation; both are much more utilized for RNA formation than are formate and glycine.* The reason for the large RNA/DNA specific activity ratio observed after administering adenine and phosphate is not small utilization for DNA formation but rather an unusually large utilization for RNA formation. If the soluble nucleotides are intermediates for nucleic acid synthesis, it would be expected that formate or glycine incorporation into acid-soluble adenylic acid nucleotides would be lower than adenine incorporation, which probably occurs at an accelerated rate. As has been pointed out, the incorporation of formate and glycine is apparently inhibited when it is administered concurrently with adenine, and it would be expected that a corresponding diminution of formate or glycine incorporation would be found in the acid-soluble adenine nucleotides.

In vitro studies have shown that more 4-amino-5-imidazole carboxamide is used in liver homogenates than in other rat tissues.[52] Whole-animal experiments in which its distribution could be determined in nucleotides and nucleic acids would be of considerable value in testing the suggestion that nucleotides are a pool for nucleic acids. One might expect to find similarities in the metabolism of 4-amino-5-imidazole carboxamide and that of orotic acid. In vitro, adenine causes an inhibition of carboxamide utilization.

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* It can be calculated from the data presented by Tyner et al.[11] that only a small fraction of the glycine administered enters into the nucleic acid of the liver. However, the total calculated for glycine and phosphate utilization may be low, as the amount of nucleic acid isolated per gram of liver appears to be much lower than would be expected on the basis of other data.[4, 16, 49, 50] Experiments with sucrose homogenates of mouse liver have indicated that extensive ribonuclease action may take place resulting in the formation of nucleic acid fragments that are not precipitated by dilute perchloric acid,[51] and this may account for low results.

If, as suggested, the acid-soluble 5'-nucleotides are intermediates in nucleic acid synthesis, one cannot overemphasize the importance of determining the incorporation of compounds that are being investigated as nucleic acid precursors in the acid-soluble nucleotide fraction as well as in the nucleic acids. This is particularly true when compounds are being administered that are rapidly excreted or extensively metabolized. Further experiments are obviously necessary to determine the incorporation of nucleotides, particularly 5'-nucleotides such as 5-AMP, into the acid-soluble nucleotides as well as into the RNA and DNA. In the past, 2'- and 3'-nucleotides[53, 54] have been administered, but, as discussed elsewhere, the emphasis should perhaps be placed on 5'-nucleotides. At the present time, further experiments are in progress to investigate by other methods the likelihood that nucleotides are used for nucleic acid synthesis. A more complete knowledge of the mechanisms by which the nucleotides are formed and utilized should aid greatly in our understanding of nucleic acid formation and renewal.

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