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INCORPORATION OF ADENINE-4,6-C114 INTO ACID-SOLUBLE NUCLEOTIDES IN THE C57 MOUSE

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ABSTRACT

Adenine-4, 6-C^{14} has been administered to male C_{57} mice, and the distribution of the radioactivity has been studied at short time intervals after administration, particularly in the cold TCA-soluble fraction. It has been shown that adenine is rapidly incorporated into the nucleotides of tissue, including 5'-adenylic acid, adenosine diphosphate, adenosine triphosphate, the corresponding guanylic acid derivatives, dephosphopyridine nucleotide, inosinic acid, and possibly TPN. The adenine is rapidly equilibrated between AMP, ADP, and ATP, and is less rapidly incorporated into DPN. The specific activity of the guanine nucleotides is always considerably less than that of the adenine nucleotides. Evidence is presented that the TCA-soluble fraction serves as a precursor for RNA and DNA.

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INTRODUCTION

The rapid and extensive metabolism of adenine in the C_{57} mouse,[1] and the evidence that has been accumulated that the compounds soluble in cold 10% trichloroacetic acid (TCA) derived from adenine may be precursors of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA),[1, 2, 3] made a more complete investigation of this fraction desirable, especially at short time intervals after administration of the adenine-C^{14}.

In the study reported here, adenine-4, 6-C_1^{14} has been administered to male C_{57} mice, and the specific activities of the adenine or guanine in the derived adenylic and guanylic acid derivatives have been determined at several time intervals. After this study had been completed, Marrian reported a similar investigation of the cold TCA-soluble adenylic acid derivatives in the rat.[4] The results are in general agreement.

METHODS

Six male C_{57} mice weighing 25 to 27 g, age 5 to 6 months, were each injected intraperitoneally with 1.3 mg of adenine-4, 6-C_1^{14} containing 2.2×10^7 dis/min, dissolved in 0.5 ml of 0.9% NaCl. At 1/2 hour, 2 hours, and 24 hours after administration of the adenine, two mice were sacrificed by decapitation and cold 10% TCA extracts were made of the small intestine (rinsed with saline), liver, kidney, and skinned carcass. The RNA and DNA fractions were isolated, and the total and specific activities were determined as previously described.[2]

A known aliquot portion of the cold 10% TCA extract was extracted with ether to remove TCA, and the total activity and "total 5-AMP" specific activity were determined in the aqueous phase.[2] The remainder of the

combined cold TCA extract from each tissue (1/6 of the carcass extract) was passed through a column containing 5 ml of acid-washed Darco G-60 charcoal. The column was washed with cold water until neutral. Only 2 to 3% of the radioactive material was not retained on the column during the adsorption and washing process. The radioactive compounds were eluted with 50 ml of 10% pyridine in water, and the eluate, which contained 80 to 90% of the radioactivity initially present, was freeze-dried. When the eluate had been concentrated to 4 to 5 ml, it was thawed and transferred to 12-ml centrifuge tubes, and the freeze drying was completed. The residue was dissolved in 0.2 ml of water, and duplicate portions were placed on oxalic acid–washed Whatman No. 4 filter paper.[5] One of each pair of papers was chromatographed two-dimensionally to the edges in 40% butanol–25% propionic acid–35% water (wt. %) and then in 60% propanol–30% ammonium hydroxide–10% water (vol. %) (6 hours), while the other paper was chromatographed for 24 hours in each direction with twice as much solvent (200 ml). All of the radioactive compounds present in the freeze-dried extract remain on the paper in the first procedure, while the second procedure separates the phosphorylated nucleotides. Radioautographs were made of each chromatogram in order to locate radioactive compounds. The uv-absorbing compounds could be located by viewing under uv light. The approximate relative percentages of the radioactive compounds were determined by counting the radioactive areas on the paper with a thin-mica-window Geiger tube.

The phosphorylated nucleotides were separately eluted from the papers with 0.1% formic acid, and the eluates were evaporated to dryness after the addition of 0.1 vol of conc HCl. The residue from each area was taken up in 4% acetic acid, placed on Whatman No. 1 filter paper, and rechromatographed with the above solvent systems used in reverse order. The adenine, guanine or hypoxanthine was located under uv light and eluted with 0.1% formic acid, and the specific activities were determined.

The adenine specific activity was determined as described.[1] Hypoxanthine was spectrophotometrically determined by the increased uv absorption at 292 mμ upon the addition of xanthine oxidase.[6] Guanase (from rat liver) and xanthine oxidase were used to oxidize guanine to uric acid, and the increased absorption at 292 mμ was used to determine the amount of guanine present.[6]

RESULTS

In this study the distribution of adenine-C^{14} in compounds extractable by

cold 10% TCA has been studied at periods ranging from 1/2 hour to 24 hours after intraperitoneal administration of 1.3 mg of adenine-4, 6-C_1^{14} to C_{57} male mice.

In Fig. 1 are shown the radioautographs obtained from 6 chromatograms of the cold TCA extract of the small intestine 1/2 hour, 2 hours, and 24 hours after administration of the adenine-4, 6-C_1^{14}. The approximate distribution of the radioactivity present on each paper after chromatography is indicated. It is readily apparent that the adenine has been extensively converted into phosphorylated derivatives such as AMP (5'-adenylic acid), ADP (adenosine diphosphate), ATP (adenosine triphosphate), and inosinic acid (IMP) within 1/2 hour after administration. The three other major radioactive compounds present are adenine (33%), allantoin (5%), and an unidentified uv-absorbing compound, "X-1" (2%). Minor amounts of the three guanylic acid derivatives--5'-guanylic acid (GMP), guanosine diphosphate (GDP), and guanosine triphosphate (GTP)--were present. The identity of the three guanylic acid derivatives has been established only by their relative positions on the paper chromatogram and by the observation that guanine is obtained upon acid hydrolysis. The identification of these areas as GMP, GDP, and GTP seems reasonable because these compounds have been isolated from rat tissue and were shown to be 5'-ribose derivatives of guanine.[7] In addition, a radioactive adenine derivative was isolated near the GTP area. It is believed to be TPN (triphosphopyridine nucleotide), from comparison of its R_f with that of an authentic sample (Sigma), but since the amount present was very small, its identity is less certain than that of DPN (diphosphopyridinenucleotide), which was found and identified in a similar manner. The quantity of DPN was estimated to be approximately 0.5 mg/g of mouse liver and 0.2 mg/small intestine by an adaptation of the glucose dehydrogenase method[8] applied to the freeze-dried eluate.* Small quantities of nonradioactive uridylic acid were often isolated upon acid hydrolysis and rechromatography of eluted uv-absorbing areas, particularly that occupied by IMP.

Table I summarizes the distribution of radioactivity in the cold TCA fraction, the RNA, and the DNA of the small intestine, liver, kidney and carcass 1/2 hour, 2 hours, and 24 hours after the administration of adenine-4, 6-C_1^{14}. These data are similar to those previously presented.[1,2] In addition, the approximate distribution of the radioactivity in the cold TCA-soluble fraction

*The authors are indebted to Mr. Robert Bartsch, who suggested the method and provided the purified glucose dehydrogenase.

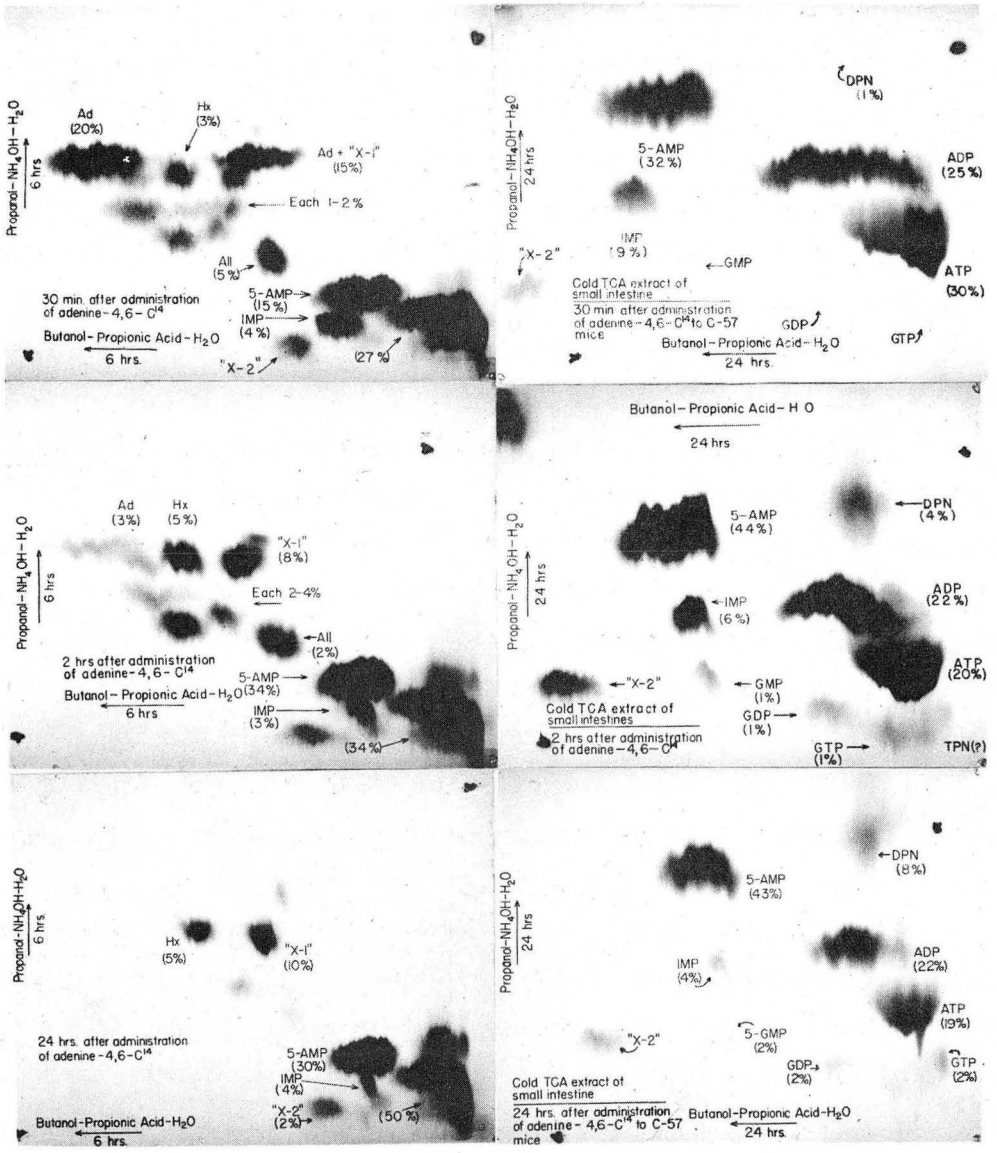

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Fig. 1. Radioautograph of two-dimensional chromatograms of the cold TCA extract of the small intestine of mice sacrificed 1/2 hour, 2 hours, and 24 hours after administration of adenine-4, 6-C^{14}. The approximate percentage distribution of radioactivity on each chromatogram is indicated by the number in parentheses.

Table I

Distribution of Adenine-4,6-C_1^{14} after Administration to C_{57} Male Mice*

Fraction	Small Intestine 1/2 hr	Small Intestine 2 hr	Small Intestine 24 hr	Liver 1/2 hr	Liver 2 hr	Liver 24 hr	Carcass 1/2 hr	Carcass 2 hr	Carcass 24 hr	Kidney 1/2 hr	Kidney 2 hr	Kidney 24 hr
	% Distribution of Injected Radioactivity											
Cold TCA	9.1	8.8	3.1	8.4	10.7	5.0	25	10	8.0	6.4	9.3	5.0
RNA	0.4	1.7	2.3	0.16	0.7	1.7	0.3	1.0	1.2	0.02	0.08	0.3
DNA	0.05	0.45	1.2	< 0.01	0.02	0.02	0.1	0.6	1.0	< 0.01	< 0.01	< 0.01
	% Distribution of Cold TCA-Soluble Radioactivity											
Nucleotides	46	70	85	50	85	95	24	> 90	> 95	15	25	40
Adenine	32	3	0	34	1	0.5	65	1	0	39	1	1
Hypoxanthine	3	5	5	2	3	0.5	2	3	1	7	3	2
Allantoin**	5	2	1	3	2	0.5	3	2	1	28	56	48
"X-1"	3	8	8	2	2	1.5	1	0	0	1	1	1
Minor Cmpds.	11	12	1	8	6	2	5	0	0	—	—	—
	% Distribution of Nucleotide Radioactivity											
5-AMP	33	44	43	20	33	23	13	15	10	40	50	40
DPN	1	4	8	4	5	9	2	6	8	--	10	17
ADP	25	22	22	34	27	30	20	26	26	10	10	12
ATP	30	20	19	33	20	21	53	40	47	20	12	17
GMP, GDP, GTP	1	3	6	2	4	10	1	1	2	0	0	7
IMP	9	6	4	7	10	8	10	11	8	30	15	7
TPN**	< 1	—	—	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1

* 1.3 mg of adenine-4,6-C_1^{14}, specific activity 17,000 dis./μg, was administered intraperitoneally to male C_{57} mice.

** Other compounds may be present in the radioactive allantoin area, particularly in the kidney. The TPN area had an R_f less than ATP in both solvents and has not been otherwise identified except by its approximate correspondence to the R_f of an authentic sample of TPN.

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has been estimated by counting the radioactive areas on the chromatograms that were run to the edge (6 hours). In the next group of data are presented the approximate distributions of the radioactivity within the nucleotides, obtained by counting the radioactive areas on the papers that were chromatographed for 24 hours in each direction.

With the exception of the "minor compounds," which have not been identified yet, the remainder of the radioactive compounds present at 1/2 hour and 2 hours after the administration of the adenine consisted of the normal nucleotide compounds present in the tissue including AMP, ADP, ATP, DPN, TPN (?), GMP, GDP, GTP and IMP, adenine which was rapidly utilized, and hypoxanthine or allantoin, which are catabolites of adenine in the mouse. By the isolation procedure used, no indications of any dinucleotides were found, and it appears unlikely that they are present in mouse tissue in significant amounts. It is possible that small amounts of such compounds would be lost in the isolation procedure. An estimated 10 to 20% of the radioactive material was not recovered from the Darco G-60 column, but this is less than the losses reported when ion-exchange resins were used for a similar separation.[4]

The specific activities of the adenine of the AMP, ADP, ATP, DPN and TPN (?), guanine of the GMP, GDP, and GTP, and hypoxanthine of the IMP were obtained after elution, hydrolysis to the purine, and rechromatography. The specific activities of the "free" adenine and "free" hypoxanthine were determined after elution from the original chromatograms. Usually several sections of the AMP, ADP, and ATP areas were eluted and analyzed separately, and the specific activities were found to be similar ($\pm$5%), thus indicating that the specific activity of each area was relatively uniform. Because of the small quantities and low specific activity, the determination of the guanine nucleotide specific activity is less reliable than that of the adenine nucleotides. Therefore no conclusions can be drawn from differences of specific activity of the guanylic acid derivatives in a given tissue sample until further experiments are done. The results are summarized in Table II.

The adenine of the 5-AMP, ADP, and ATP was found to have essentially equal activities in the individual tissues even 1/2 hour after administration of the adenine, at which time free adenine is still present. The different specific activities of the carcass AMP, ADP, and ATP are believed to represent the two major tissue pools from which these nucleotides are isolated. The muscle contributes the major portion of the ADP and ATP with a lower renewal rate or specific activity than the AMP, which is believed to be predominately from

Table II

Specific Activity in Dis /μg of Soluble Nucleotides, RNA, and DNA Derived from Adenine-4,6-C_1^{14} in Mice*

	Carcass			Small Intestine			Liver			Kidneys		
	1/2 hr	2 hr**	24 hr	1/2 hr	2 hr	24 hr	1/2 hr	2 hr	24 hr	1/2 hr	2 hr	24 hr
"Total 5-AMP"***	156	215	178	1810	3340	1920	820	1690	1500	615	1400	1080
5-AMP	425	550	300	2135	3130	2070	1100	2250	1430	990	1900	1230
DPN	24	108	200	320	1780	1890	103	645	1585	--	350	1020
ADP	165	224	178	2220	3060	1725	1060	1900	1390	705	1620	1015
ATP	155	198	197	1950	3440	1145	1050	1720	1410	690	1530	960
TPN (?)	148	160	189	420	--	--	250	600	1750	60	295	1250
IMP	150	155	142	2740	2660	1210	825	1540	1100	650	1380	1250
GMP	182 (?)	185	95	190	325	265	19	315	330	34	330	530
GDP	86	136	50	180	400	200	20	215	335	---	---	---
GTP	47	89	53	285	535	190	30	180	310	---	---	---
Hypoxanthine	~1500	1230	645	2520	1960	1150	~3000	3240	2760	~2000	2610	1480
Adenine	14,000	1430	250	15,000	9350	---	15,500	~2400	~1150	17,300	8200	----
RNA-Adenine												
Mouse A	57	165	168	130	401	670	32	134	385	48	214	500
Mouse B	50	202	187	132	350	760	48	166	368	41	215	470
DNA-Adenine												
Mouse A	16.8	103	146	17.8	124	391	3.6	15	11	<1	1.7	9.0
Mouse B	17.7	130	163	16.6	116	450	1.3	22	13	<1	1.1	7.5
RNA/DNA	3.1	1.6	1.15	7.7	3.2	1.7	1.6	8	31		150	60

(*) 1.3 mg. of adenine-4,6-C_1^{14}, specific activity 17,000 dis /μg, was administered intraperitoneally to male C_{57} mice. All results are expressed in dis /μg purine, calculated as adenine equivalent.

(**) Time after administration of adenine-C_1^{14}.

(***) "Total 5-AMP" refers to the 5-adenylic acid obtained upon hydrolysis of the cold TCA extract to 5'-adenylic acid with $Ca(OH)_2$.

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the bone marrow with a much more rapid renewal.

The specific activity obtained for DPN was significantly lower than that obtained for the "total 5-AMP" at 1/2 and 2 hours after administration of the adenine, which indicates that its renewal from ATP and nicotinamide ribotide[9,10] was slow as compared to the AMP⇌ADP⇌ATP transformations. The fraction believed to be TPN had specific activities very similar to DPN and much lower than the AMP, ADP, and ATP, indicating that it was indeed a separate compound. A TPN fraction was obtained for the small intestine only at 1/2 hour with specific activity similar to that obtained for DPN. The specific activity of the TPN (?) of the carcass was about equal to the ADP and ATP, but the possibility of incomplete separation of the nucleotides was greater with these chromatograms. In addition, the inclusion of two metabolically very different tissues in this fraction makes the result difficult to interpret. The possibility exists of the presence of another adenylic acid derivative such as the adenosine tetraphosphate reported by Marrian.[11]

The inosinic acid of the carcass had essentially the same specific activity as the ADP and ATP, indicating that it was probably derived primarily from the muscle adenylic acid. With the exception of the one value obtained for the IMP of the small intestine at 1/2 hour, IMP values were lower than the corresponding AMP specific activity. This is evidence that the IMP is derived from, rather than a precursor of, 5-AMP. The relatively high specific-activity values obtained for the hypoxanthine would indicate the existence of a more direct route for the conversion of a portion of the adenine to hypoxanthine, probably by the direct action of adenase.

The specific activity of the guanylic acid derivatives was much less than the corresponding adenylic acid derivatives, but the results indicate that adenine may be converted to guanine at the nucleotide level. Other investigations have shown that labeled guanine was present in the RNA and DNA after labeled adenine had been administered to rats[12] or mice,[1] but the conversion of adenine into acid-soluble guanylic acid derivatives has not been previously shown.

In this experiment, the RNA and DNA from the tissue of each mouse were isolated separately. The values are listed individually in Table II. The agreement between duplicate mice was good; values seldom differed by more than 15%. In all tissues, the RNA became radioactive within 1/2 hour after administration of the adenine. As previously reported,[1,2] the specific activity of the nucleic acid adenine is higher 24 hours after administration

of the adenine than at 2 hours, a time at which essentially all of the free adenine has been converted to other compounds. More of a "lag" is evident in the uptake of the adenine into the DNA than is evident for the incorporation of the adenine into the RNA, as seen by comparison of the RNA/DNA specific-activity ratio at 1/2 hour to that at 2 hours or 24 hours.

DISCUSSION

Evidence has been presented that adenine is incorporated into compounds in the cold TCA fraction which are subsequently incorporated into RNA and DNA in both mice and rats.[1,2,3] Orotic acid has been shown to be incorporated in the liver of the rat into uridylic acid derivatives, which are subsequently converted into RNA uridylic acid.[13] In the previous paper[2] evidence was presented that the "total 5-AMP" of the small and the large intestine and perhaps also of the carcass was in relatively rapid equilibrium with the RNA fraction. In this study it has been shown that the major compounds into which the adenine is incorporated are the adenine nucleotides, AMP, ADP, ATP, and--to a lesser extent--DPN and perhaps TPN. The specific activities of the AMP, ADP, and ATP are very similar even shortly after administration of the adenine in a given tissue. This is in agreement with the observations on the turnover of P^{32} in the nucleotides of the skeletal muscle of the rabbit, in which it was found that the terminal P atom of ATP was replaced 6 to 8 times as rapidly as the middle P atom, and this in turn was replaced about 16 times as rapidly as the P atom attached to the ribose moiety of 5-AMP.[14] Similar results were found in the cat, where it was also shown that the equilibration of P^{32} was much more rapid in the liver and kidney than in the muscle.[15] These results indicate that once the adenine is incorporated into adenylic acid, its conversion into ADP and ATP is very rapid. The specific activities of the AMP, ADP, and ATP reported by Marrian[4] show considerably larger differences than those which were observed in this study, and larger than would be expected on the basis of the P^{32} data from other animal studies. The results presented by Marrian[4] indicate comparable percentage conversion of the adenine into the nucleotides of the rat in Experiment 6 and apparently somewhat lower in Experiment 7. The specific activities of the RNA and DNA adenine were not presented in this series of experiments, so no comparison of these values, which would be of considerable interest, can be made.

Orotic acid, when administered to a rat, is rapidly converted into several uridine nucleotide derivatives which become uniformly labeled within one hour.[13] It has also been shown[16] that the 5'-mono-, di-, and triphosphates of any given nucleotide isolated from Flexner-Jobling rat carcinoma have similar specific activities within one hour after administering glucose-1-C^{14}.

No evidence was found for the presence of a highly radioactive adenine nucleotide compound of the type suggested by Oldwasser from in vitro experiments with pigeon liver homogenates.[17] It is possible that small amounts of adenine nucleotides such as these found by Hurlbert et al.[18] may be present.

Guanine nucleotides have been shown to occur in the cold acid-soluble fraction of rat[7] and mouse ascites tumors,[19] but the occurrence in normal mouse tissue has not previously been shown. Guanylic acid derivatives were not found by the dimethylformadine-extraction-paper-chromatographic separation procedures reported by Dorough and Seaton.[20] The relatively low specific activity of the guanylic acid derivatives would indicate either that their renewal is slow or that other major mechanisms of synthesis exist. This latter possibility is preferred because the renewal of nucleic acids in these tissues is believed to be rapid in comparison to the size of the pool of guanylic acid derivatives, and thus, if the 5'-nucleotides are intermediates in the formation of RNA and perhaps DNA, the turnover rate of the soluble nucleotides should be high. In addition, it has been shown[21] that the specific activity of nucleic acid guanine is higher than nucleic acid adenine after administration of glycine, thus indicating that a larger proportion of the guanine is synthesized de novo.

The rapid utilization of adenine to form nucleotides has been shown in perfused rabbit* and cat livers.** The conversion of adenine into nucleotides has been demonstrated in cell-free pigeon liver homogenates.[17] Saffron and Scarano presented evidence for a "nucleotide phosphorylase" which catalyzes the condensation of ribose phosphate (believed to be ribose-1, 5-diphosphate) with adenine to form adenylic acid.[22] They obtained the enzyme in a particulate-free solution when pigeon livers were homogenized in a potassium chloride-phosphate buffer. Preliminary experiments indicate

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* E. L. Bennett and H. M. Kalckar, unpublished.

** E. Goldwasser and H. M. Kalckar, unpublished.

that if the homogenization and particulate fractionation are carried out in 0.25 M sucrose,[23] two fractions, the mitochondrial fraction and the cytoplasmic supernatant fraction, are required to form adenylic acid from adenine.* An enzyme has been isolated from yeast which catalyzes the condensation of adenine with 5'-phosphoribosylpyrophosphate to yield adenylic acid.[24] A similar enzyme system is probably operative in animal tissue. As has been discussed,[2] the rate of uptake of adenine is more than would be calculated from the rate of disappearance of the radioactive "total 5-AMP" from the tissue. It may represent increased net synthesis of adenylic acid due to the abnormally large amount of adenine present shortly after it has been administered. At short time intervals, there appears to be an increased quantity of inosinic acid present, indicating that the organs are using the conversion of adenine to adenylic acid and then inosinic acid as a "detoxification" mechanism. The amount of adenase in the mouse is small. Direct oxidation of the adenine to 2,8-dioxyadenine produces a highly insoluble and therefore toxic compound.[25] In contrast, the mouse can directly convert a relatively large amount of guanine to allantoin. This may, in part, explain the large difference in degree of utilization of injected adenine and guanine. Another factor may be the size of the nucleotide pool. A second mechanism that could be postulated for the incorporation of adenine into adenylic acid would be a trans-N-glycosidase type of reaction, which has been reported for desoxyribosides but which has not, as yet, been shown for ribosides or ribotides.[26] This would involve no net synthesis of nucleotide but would be merely an exchange reaction.

Evidence has been presented in previous papers[1,2,3] that compounds other than free adenine that are present in the cold acid-soluble fraction serve as precursors for both RNA and DNA. The most attractive suggestion is that these compounds are the adenine nucleotides, AMP, ADP, and ATP. The experiments of Dancis et al.[27] would appear to rule out the catabolites of nucleic acids, i.e., allantoin and perhaps hypoxanthine, as precursors of nucleic acids. The results presented here indicate that nucleotides are the major products formed that remain in the tissues after administration of adenine, but until all of the minor components have been identified the possibility cannot be completely excluded that some of these may be intermediates, perhaps even trace amounts of the imidazole-carboxamide

* E. L. Bennett and B. Krueckel, unpublished.

ribotide. Present evidence, however, indicates that the adenine ring, when once formed, is not reopened;[28] experiments are now in progress to test this concept.

No evidence has been obtained for any radioactive area on the paper chromatograms which upon hydrolysis would give two different purine or pyrimidine bases, thus the existence in tissue of mixed dinucleotides, which are nucleic acid precursors or intermediates, does not seem likely. However, such compounds might exist in very small amounts and might be included in the 10% to 20% of the radioactivity that was not recovered from the Darco columns. It would be desirable to test the isolation procedure with added dinucleotides.

A more detailed discussion of nucleotide-nucleic acid interrelationships is presented in the preceding paper.[2]

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