CONCERNING THE METABOLISM OF ADENINE BY THE RAT

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The metabolism of both carbon and nitrogen labelled adenine by the rat has been extensively studied by BROWN and his colleagues. Thus it was shown that adenine could be utilised for the synthesis of polynucleotide purines, that it was extensively catabolised and excreted as allantoin[1], that high doses caused kidney damage accompanied by deposition of 2:8-dihydroxyadenine[2], but that, surprisingly enough, adenine did not seem to be used as a precursor of muscle adenosine-5'-triphosphate (ATP)[1]. The differential labelling of polynucleotide purines in ribo- and desoxyribonucleic acid in the various visceral organs of the rat[3] showed that polynucleotide synthesis from adenine was limited to the ribonucleic acid unless the organ was undergoing cell division, when desoxyribonucleic acid was also formed.

Apart from the muscle ATP referred to above, none of the acid-soluble nucleotides of rat viscera had been investigated as adenine metabolites when this work was begun. Since then, GOLDWASSER[4] has described some experiments on cell free pigeon liver homogenates, and a fuller account by BENNETT[5] shows a general picture of adenine metabolism by the C57 mouse. A preliminary announcement of the present work has also appeared[6].

METHODS

Adenine-8-^{14}C was prepared by the method of CLARK AND KALCKAR[7]. ABRAMS AND CLARK[8] have commented on this method of preparation of 8-labelled adenine and their criticisms are confirmed in that the isotopic yield from sodium formate was *ca.* 50%.

Ultraviolet measurements were made on a Unicam SP 500 spectrophotometer. Concentrations of substances were computed using the following molar extinction coefficients at 260 mμ and pH 2 (0.01 *N* HCl or formic acid); adenine, 12,800; guanine, 8,200; adenosine phosphates, 14,200; (Private communications from Dr. W. E. COHN. See also COHN[9] and COHN AND CARTER[10]).

Radioactivity measurements were made on a windowless gas-flow (methane) counter operating in the proportional range as described by TAYLOR AND SHARPE[11]. Solutions to be counted were plated out on aluminium planchets in decreasing volumes until the calculated specific activities (counts per minute per μmole) agreed for each volume (means varying by less than 5% from extremes) showing negligible loss by self-absorption. Backgrounds and samples were counted sufficiently long to give a standard error of less than 4% in almost all cases. Two or three fractions were counted from each ion-exchange elution band to confirm the constancy of the specific activity.

Phosphorus analyses — by the method of ALLEN[12].

Pentose analyses — by the modified[13] method of MEJBAUM[14].

Ion-exchange resins. Dowex 1 and 2 (anion exchanger) and Zeokarb 215 (cation exchanger) were prepared for use by conventional methods of copious solvent extractions.

"Cold" — means 0–4° C

Energy-rich phosphate in the adenosine di- and tri-phosphate fractions was kindly estimated by Dr. E. C. SLATER[15] of the Molteno Institute, University of Cambridge.

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Injection of adenine and treatment of tissues. All injections were intraperitoneal at doses approximating to 0.2 mmole/Kg (see Table headings). The acid-soluble nucleotides were extracted from the tissues investigated with cold trichloracetic acid (SCHMIDT AND THANNHAUSER[16]) and either precipitated from the neutralised extract by addition of mercuric acetate solution (Exp. 2, 3, 5, 6, 7) or freed from trichloracetic acid by continuous ether extraction (Exp. 1, 2, 4). Treatment of the mercuric precipitate (which contained more than 98% of the radioactivity associated with the trichloracetic acid extract) by H_2S followed the usual methods. Anion-exchange separations of the so obtained acid-soluble fractions either utilised small (3 × 1 sq. cm) columns using chloride eluants (COHN AND CARTER[10] (Exp. 1, 4) or formic acid/ammonium formate eluants (Exp. 2, 3, 5, 6) (0.1*M* each, 0.3*M* each and 0.6*M* each removed, in turn, adenosine mono-, di- and tri-phosphates) or the longer (10 × 1 sq. cm) columns (Exp. 6, 7). The solvents used in the latter case were 0.002*N* HCl, 0.01*N* HCl/0.025*M* NaCl and 0.01*N* HCl/0.1*M* NaCl.[17] Acid hydrolysis of the acid-soluble fractions (Exp. 1, 2, 4) was by hot 72% perchloric acid (MARSHAK AND VOGEL[18]) followed by paper chromatographic separation using the *iso*propanol/HCl solvent described by WYATT[19]. Extraction of sodium nucleates (Exp. 1) by the method of PLENTL AND SCHOENHEIMER[20] was followed by alkaline hydrolysis (*ca.* 1 ml 1*N* KOH/100 mg sodium nucleate at room temperature for 24 hours). Neutralisation with cation exchanger and acidification to pH 4 with dil. HCl precipitated the DNA fraction. Acid hydrolysis formed free purines from the ribonucleotide supernatant which were separated on paper as before.

RESULTS

TABLE I

Exp. 1. Approximate specific activity of adenosine phosphates from the acid-soluble fraction of rat liver and of ribonucleic acid purines 2 hours after injection (0.20 mmole/kg) of adenine-8-^{14}C ($6.86 \cdot 10^3$ cpm/μmole): 1 rat — 445 g.

Adenine nucleotide from acid solubles	*Specific activity*
(Adenine — by hydrolysis	850)
Adenosine monophosphate fraction	420–880
Adenosine diphosphate fraction	460–835
Adenosine triphosphate fraction	490–560
Ribonucleic acid purines	
Adenine	*ca.* 44
Guanine	*ca.* 12

The wide variation in the specific activities recorded above was due to the presence of non-ultraviolet absorbing contaminants. This effect was eliminated by the preliminary mercuric acetate precipitation carried out in the rest of the experiments.

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TABLE II

Exp. 2. Specific activity and relative analysis of adenosine phosphates from the acid soluble fraction of rat organs 2 hours after injection (0.20 mmole/kg) of adenine-8-^{14}C ($9.90 \cdot 10^3$ cpm/μmole): 3 rats (960 g).

LIVER

Adenine nucleotide	*Adenylic acid*	*Pentose*	*Total organic phosphorus*	*Easily hydrolysable phosphorus*	*Specific activity*
(Adenine — by hydrolysis	—	—	—	—	720)
Adenosine monophosphate fraction	1.00	1.1	—	0.0	850
Adenosine diphosphate fraction	1.00	1.2	—	1.0	730
Adenosine triphosphate fraction	1.00	1.1	3.2	1.1	600–740

Recoveries. 3.1 μmole adenosine phosphate/g fresh liver representing 50% of the optical density at 260 mμ and 69% of the radioactivity of the acid-soluble extract precipitated as mercuric salt.

REST OF VISCERA

(Adenine — by hydrolysis	—	—	—	—	970)
Adenosine monophosphate fraction	1.00	0.9	—	0.0	1240
Adenosine diphosphate fraction	1.00	1.2	—	1.0	770
Adenosine triphosphate fraction (very small amount)	—	—	—	—	*ca.* 400

Recoveries. 1.4 μmole adenosine phosphates/g fresh viscera representing 61% of the optical density at 260 mμ and 58% of the radioactivity of the acid-soluble extract precipitated as mercuric salt.

TABLE III

Exp. 3. Specific activity of adenosine phosphates from the acid-soluble fractions of rat liver and rest of viscera 2 hours after injection (0.22 mmole/kg) of adenine-8-^{14}C ($2.35 \cdot 10^4$ cpm/μmole): 1 rat — 338 g.

Adenine nucleotide	*Specific activity*	
	Liver	*Rest of viscera*
Adenosine monophosphate fraction	4620	6450
Adenosine diphosphate fraction	3630	5550
Adenosine triphosphate fraction	Only a trace isolated	None

TABLE IV

Exp. 4. Specific activity of adenosine phosphates from the acid-soluble fraction of rat liver 24 hours after injections (0.19 mmole/kg/day for 3 days) of adenine-8-^{14}C ($6.33 \cdot 10^3$ cpm/μmole): 1 rat — 345 g.

Adenine nucleotide	*Specific activity*
(Adenine — by hydrolysis	1190)
Adenosine monophosphate fraction	1070
Adenosine diphosphate fraction	1150
Adenosine triphosphate fraction	885

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TABLE V

Exp. 5. Specific activity of adenosine phosphates from the acid-soluble fraction of rat viscera and of rat muscle 2 hours after injection (0.24 mmole/kg) of adenine-8-^{14}C ($1.47 \cdot 10^5$ cpm/μmole): 1 rat — 308 g.

Adenine nucleotide	*Specific activity*	
	Viscera	*Muscle*
Adenosine monophosphate fraction	24,750	—
Adenosine diphosphate fraction	20,300	830
Adenosine triphosphate fraction	5,080	700–1000
Analysis of the adenosine triphosphate fractions (molar ratios)		
Adenylic acid	1.00	1.00
Easily hydrolysable phosphorus	0.9	2.0

TABLE VI

SPECIFIC ACTIVITY OF ADENOSINE PHOSPHATES FROM THE ACID SOLUBLE FRACTIONS OF SEPARATE RAT ORGANS 2 HOURS AFTER INJECTION (0.20 mmole/kg) OF ADENINE-8-^{14}C (3.77 and $7.55 \cdot 10^4$ cpm/μmole FOR EXP. 6 AND 7, RESPECTIVELY). :— 6 rats — 1551 g; and 9 rats — 1037 g

Exp.	*Liver*			*Intest.*			*Spleen*			*Kidney*			*Testis*		
	AMP frac.	*ADP frac.*	*ATP frac.*	*AMP frac.*	*ADP frac.*	*ATP frac.*	*AMP frac.*	*ADP frac.*	*ATP frac.*	*AMP fra .*	*ADP frac.*	*ATP frac.*	*AMP frac.*	*ADP frac.*	*ATP. frac.*
6	5100	3550	3800	5780	4590	1330	3180	3170	—	5730	—	—	830	815	—
7	5880	4120	1950	7540	—	—	5700	—	—	4840	1800*	—	860	620	—

* This material, eluted in the position usually occupied by the diphosphate fraction, had absorption maximum at 255 mμ and presumably contained little adenine nucleotide.

TABLE VII

ENZYMIC ASSAY OF ADENOSINE DI- AND TRI-PHOSPHATE FRACTIONS FOR ADP AND ATP

	Concn. adenosine phosphate	*Concn. of energy-rich phosphate*
Adenosine diphosphate fraction	0.98 μmole/ml	0.56 μmole/ml
Adenosine triphosphate fraction	0.55	0.04

DISCUSSION

In general, it is immediately obvious that the injected adenine is metabolised extensively to the acid-soluble nucleotides from the internal organs of the rat (Tables I–VI). This confirms in the rat, the relevant results of GOLDWASSER[4] and BENNETT[5] in different biological systems.

The structure of these nucleotide fractions is not clear. While it seems very probable that the monophosphate fraction contains mainly the well-known adenosine-5′-phosphate, the composition of the di- and tri-phosphate fractions is by no means certain. GOLDWASSER[4] has presented evidence that the content of ADP and ATP in his di- and

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tri-phosphate fractions was less than would appear from the ultraviolet measurements. These findings have been confirmed by the present author (Table VII) and in addition, the content of easily hydrolysable phosphorus in the triphosphate fraction (when enough has been present to analyse) has also been inconsistent with the concentration of ATP measured spectrophotometrically (Tables II and V). It follows, therefore, that to continue to refer to these fractions as ADP and ATP when, in fact, all that is certain is that they are adenine pentosides containing 2 and 3 phosphoric acid residues per mole of base is to risk confusion. Thus, in the Tables presented here, the designation of a nucleotide fraction as "adenosine diphosphate fraction" is to emphasise this lack of precise knowledge.

HEINRICH AND WILSON[21] have shown that the usual purine precursors (carbonate, glycine, formate) gave rise to acid-soluble nucleotides in the rat. However, during the course of their experiments which ranged from 6 hours to 10 days, it appeared that the adenine of the acid-soluble nucleotides was in fairly rapid equilibrium with the ribonucleic acid adenine, since the specific activities of both were almost the same. A similar equilibrium was set up when 4-amino-5-imidazole-carboxamide was administered to rats over 3 days at 3.2 mmole/Kg/day[22]. The present work does not confirm this rapid equilibrium, since the specific activity of the acid-soluble nucleotides was much higher than the ribonucleic acid adenine after 2 hours (Table I) and after 24 hours[23]. It is interesting that BENNETT'S figures[5] show similar results in viscera, but that the values quoted for carcass imply a rapid equilibrium. Again, the facile synthesis of muscle ATP from formate or glycine[24] contrasts with reports of the poor utilisation of adenine[1] and of ammonia[25] in this system. However, BENNETT'S results[5] would imply a much greater incorporation into muscle ATP than BROWN obtained, and Table V, although only representing a single experiment, confirms this.

In Tables II and III, the general results for liver are in agreement with those of Table I in that no obvious variation in the specific activities of the three nucleotide fractions is noticeable. The values for the rest of the viscera, however, suggested that one of the organs might well be giving rise to a more highly labelled monophosphate fraction, but this contention could not be clearly upheld by the separate organ experiments shown in Table VI.

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SUMMARY

Radioactive adenine has been injected into white male rats and the distribution of radioactivity in the acid-soluble nucleotide fractions of various organs has been examined.

RÉSUMÉ

L'adénine radioactive a été appliquée à des rats blancs masculins. La distribution radioactive dans les organes due aux diverses fractions de nucléotides, soluble en acides, a été examinée.

ZUSAMMENFASSUNG

Radioactives Adenin wurde männlichen weissen Ratten injiziert. Die Verteilung der erhaltenen Radioaktivität in den säurelöslichen Nucleotidfraktionen von verschiedenen Organen wurde untersucht.

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E. E. B. SMITH, A. MUNCH-PETERSEN AND G. T. MILLS (*Nature*, 172 (1953) 1038) state that large quantities of acid-soluble uracil nucleotides occur in rat liver. In order that the specific activities recorded in the present paper be not called in doubt, it is necessary to advance reasons for believing that the fractions isolated contained negligible amounts of uracil nucleotides.

1. When the acid-soluble fraction, with or without mercuric precipitation, was hydrolysed by hot perchloric acid and the liberated bases separated on paper, only traces of ultraviolet-absorbing materials other than adenine were detected.

2. One half of an acid-soluble extract of rat liver, freed from acid by ether extraction was submitted to chloride elution from a 10 × 1 sq. cm column of Dowex 2. After elution of the adenosine monophosphate fraction with 0.002 *N* HCl, no ultraviolet-absorbing material was eluted by 0.005 *N* HCl. The other half of the extract, to which was added 3.0 mg uridine-5′-monophosphate (UMP), was submitted to the same separation, when 2.98 mg UMP was eluted by the 0.005 *N* HCl.

3. In all separations which have been carried out on animal organs (including rat liver) since the appearance of the above paper, the various peaks of the phosphate fractions have been tested with bromine water. In no case has the spectrum of the solutions been destroyed or reduced in intensity by this treatment.

For these reasons, the author is confident that the specific activities reported here were not measured on solutions containing mixtures of adenine and uracil nucleotides. This belief receives confirmation from Tables I, II, and IV, where the specific activity of adenine, derived from the mixture of acid-soluble nucleotides and separated by different techniques, is in good agreement with the specific activities of these nucleotide fractions.