THE NUCLEIC ACID METABOLISM OF ANIMAL CELLS *IN VITRO*

III. FACTORS INFLUENCING NUCLEOTIDE BIOSYNTHESIS

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SUMMARY

In the Ehrlich ascites carcinoma of the mouse the rate of purine biosynthesis *in vitro* under aerobic conditions is greatly increased if the cells are supplied with glucose or uridine, although neither compound appears to increase the energy available to the cells. Glucose and nucleosides also increase the rate at which the cells take up adenine and uracil from the medium and convert them to nucleotides. Glucose has no effect on uptake of glycine and phenylalanine under anaerobic conditions, although it increases phenylalanine uptake when the cells are incubated under nitrogen. The L5178Y mouse ascites lymphoma differs from the Ehrlich ascites carcinoma in having a much higher content of glucose *in vivo*, a lower capacity for glucose uptake *in vitro*, and a higher capacity for purine nucleotide biosynthesis *in vitro*. In rabbit bone marrow, purine nucleotide biosynthesis *in vitro* is slow relative to nucleic acid biosynthesis, is not affected by the presence of glucose, and is only slightly increased by a mixture of glucose, glutamine, aspartic acid and glycine. It differs from the corresponding process in the ascites carcinoma in continuing at a steady rate for a longer period, and in being more sensitive to the inhibitory effects of azaserine. Chick embryo brain and skeletal muscle also synthesize purine nucleotides relatively slowly *in vitro*. In the latter, but not the former, the synthesis is accelerated by a mixture of glucose and glutamine.

INTRODUCTION

It has previously been shown that *in vivo* [^{14}C]formate is extensively incorporated into the nucleic acid purines and DNA thymine of rapidly-growing animal tissues. *In vitro*, however, many such tissues show only a limited incorporation of [^{14}C]formate into nucleic acid purines, although incorporation into DNA thymine remains high[1-5]. Experiments with "L" strain mouse fibroblasts grown in tissue culture have suggested

Abbreviations: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate, DPNH, reduced diphosphopyridine nucleotide; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

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that this type of incorporation *in vitro* is due to a low rate of purine nucleotide biosynthesis, so that the purine nucleotides required for the formation of new nucleic acid molecules are derived mainly from the existing unlabeled purine nucleotides of the cell, rather than from the products of current synthesis[5]. In the Ehrlich ascites carcinoma there is evidence that the low rate of purine biosynthesis found *in vitro* is due to the absence of the glucose supply which the cells normally receive *in vivo*[4,6]. Much higher rates of purine nucleotide biosynthesis can be obtained *in vitro* by addition of glucose to the incubation medium. However, in other rapidly-growing tissues purine nucleotide biosynthesis does not appear to be dependent on glucose supply. Thus the low rate of synthesis in rabbit bone marrow *in vitro* is not increased by addition of glucose. The experiments described in the present paper were undertaken to obtain more information about the factors influencing the rate of purine nucleotide biosynthesis in rapidly-growing tissues and, in particular (a) to elucidate the mechanism by which glucose affects purine nucleotide biosynthesis in the Ehrlich ascites carcinoma, (b) to determine whether the corresponding process in bone marrow is similarly dependent on an external supply of some precursor analogous to glucose, (c) to investigate the extent to which the behaviour of bone marrow or the Ehrlich ascites carcinoma is typical of other tissues whose capacity for purine nucleotide biosynthesis *in vitro* is limited.

EXPERIMENTAL

Biological material

The Ehrlich ascites carcinoma and the L5178Y ascites lymphoma[7] were kindly supplied by Dr. G. POPJAK of Hammersmith Hospital, London and Dr. G. A. FISCHER of Yale University, New Haven, Conn., respectively. The Ehrlich tumour was maintained in mice of the departmental colony and the lymphoma in DBA/2 F1 hybrid mice. C3H mice bearing a transplantable mammary adenocarcinoma were kindly provided by Dr. B. D. PULLINGER and Dr. A. HEAD of the Royal Beatson Memorial Hospital, Glasgow. This tumour originated as a spontaneous carcinoma in an ex-breeding female of the C3Hf/He strain[8,9]. For experiments on normal mouse tissues, animals from the departmental colony were used. Rat tissues were obtained from albinos of the departmental colony, weighing between 200 and 250 g. The rabbits employed were young albinos weighing approximately 1500 g. Chick embryos were obtained from eggs supplied by the Poultry Research Centre, Edinburgh.

Experimental procedures

In experiments with ascites tumours *in vitro*, tumour material from several animals was pooled, isotope was added, and 5-ml portions were dispensed into 25-ml conical flasks containing the appropriate additions. These were then incubated at 37° and simultaneously shaken at a rate of 60 to 80 oscillations/min. Bone marrow was similarly incubated as a suspension of cells[10] in Krebs–Ringer bicarbonate buffer. Other solid tissues were incubated in Krebs–Ringer bicarbonate buffer in the form of slices approximately 0.3 mm thick prepared in a MCILWAIN AND BUDDLE tissue slicer[11]. Each flask contained approx. 0.5 to 1.0 g tissue in 5 to 10 ml buffer. All incubations, unless otherwise stated, were for a period of 4 h.

Analytical methods

The analytical methods for measuring incorporation of [^{14}C]formate and other labeled precursors into nucleic acids and acid-soluble nucleotides were those previously described[3]. In experiments to determine the rate at which ascites tumour cells take up various labeled compounds from the suspension medium, the flasks and their contents were chilled in ice at the end of the incubation period. The cells were then centrifuged down, washed three times with Krebs–Ringer bicarbonate and finally disrupted by suspension in distilled water.

Chromatography

The chromatographic methods used to separate the free bases obtained on hydrolysis of nucleic acids and acid-soluble nucleotides were those previously described[3]. In experiments on uptake of [2-^{14}C]uracil the acid-soluble fraction of the cells was concentrated by lyophilization, applied to paper and chromatographed in butanol–water[12] to separate free bases and nucleosides from nucleotides. In experiments on uptake of [8-^{14}C]adenine a similar procedure was followed except that two-dimensional chromatography was used with distilled water as solvent in the first dimension and butanol–acetic acid[13] in the second.

Methods of estimation

Glucose was determined by the glucose oxidase/peroxidase method (Boehringer). Pyruvate was estimated by allowing it to react with DPNH in presence of lactic dehydrogenase and observing the resultant change in optical density at 340 mμ (Boehringer). Lactate was determined by the method of HULLIN AND NOBLE[14]. ATP was estimated by allowing it to react with 3-phosphoglyceric acid in presence of phosphoglycerokinase and measuring the 1,3-diphosphoglyceric acid produced by observing the change in O.D. at 340 mμ in presence of DPNH and phosphoglyceraldehyde dehydrogenase (Boehringer). ADP was estimated by allowing it to react with phosphoenolpyruvate in presence of pyruvate kinase and measuring the pyruvate liberated (Boehringer). AMP was estimated by converting it to ADP in presence of ATP and myokinase and estimating the ADP as described above (Boehringer). In all the Boehringer methods reagents supplied by C. F. Boehringer & Soehne, GmbH., Mannheim, Germany, were used.

RESULTS

The Ehrlich ascites carcinoma

The pattern of incorporation of [^{14}C]formate into the nucleic acid bases of the Ehrlich ascites carcinoma *in vitro* is shown in Table I. In absence of any additions, incorporation into DNA adenine is disproportionately low compared to incorporation into DNA thymine. Increased incorporation into nucleic acid adenine can be obtained by addition of glucose or uridine. This appears to be a specific effect: incorporation into DNA thymine is not affected to a comparable degree. Uracil, deoxyuridine and deoxycytidine have no effect[6]. To determine whether these effects are due to glucose and uridine acting as sources of energy, the levels of AMP, ADP and ATP in the cells were estimated (Table II). *In vivo*, the cells have a high content of ATP and relatively little AMP or ADP. Incubation *in vitro* under aerobic conditions does not materially

TABLE I

INCORPORATION OF [^{14}C]FORMATE INTO THE ACID-SOLUBLE AND NUCLEIC ACID BASES OF THE EHRLICH ASCITES CARCINOMA AND NORMAL RABBIT BONE MARROW *in vitro*

Initial isotope concentration 5 μC/ml.

Experiment No.	*Tissue*	*Additions to incubation medium*	*Special conditions*	*Specific activity (counts/min/μmole)*			
				Acid-soluble adenine	*RNA adenine*	*DNA adenine*	*DNA thymine*
1	Ehrlich ascites carcinoma	Nil	Nil	—	1,460	230	9,150
		Glucose (5.0 m*M*)	Nil	—	15,000	2,740	9,000
		Uridine (0.5 m*M*)	Nil	—	10,200	2,100	12,100
2	Ehrlich ascites carcinoma	Nil	Nil	16,680	1,130	480	9,240
		Glucose (5.0 m*M*) + glutamine (1.0 m*M*)	Nil	63,000	6,600	2,110	14,900
		Nil	Cells incubated 30 min before isotope added	10,500	590	200	9,420
		Glucose (5.0 m*M*) + glutamine (1.0 m*M*)	Cells incubated 30 min before isotope added	5,100	370	60	11,040
3	Bone marrow	Nil	Nil	—	1,130	260	6,250
		Glucose (5.0 m*M*)	Nil	—	1,010	250	6,280
		Uridine (0.5 m*M*)	Nil	—	1,910	450	9,780
		Cytidine (0.5 m*M*)	Nil	—	1,640	280	6,380
4	Bone marrow	Nil	Cells washed 3 times with buffer before incubation	4,180	4,040	460	8,150
		Glucose (5.0 m*M*), glutamine (0.5 m*M*), aspartic acid (0.5 m*M*), glycine (0.5 m*M*)	Cells washed 3 times with buffer before incubation	16,700	19,100	1,930	14,600
5	Bone marrow	Nil	Nil	88,800	7,950	790	11,100
		Nil	Cells incubated 15 min before isotope added	83,800	6,520	590	9,250
		Nil	Cells incubated 30 min before isotope added	86,000	6,470	610	9,440
		Nil	Cells incubated 60 min before isotope added	83,100	5,670	420	6,700
6	Bone marrow	Nil	Nil	—	4,190	2,720	51,500
		Nil	Cells suspended in blood serum instead of buffer	—	8,530	7,130	118,400

TABLE II

EFFECT OF INCUBATION *in vitro* ON AMP, ADP AND ATP CONTENT OF ASCITES TUMOURS

Tumour	*Additions*	*Time (min)*	*AMP µmole/ml*	*ADP µmole/ml*	*ATP µmole/ml*
Ehrlich ascites	Nil	0	0.101	0.168	0.545
Carcinoma	Nil	60	0.049	0.125	0.504
(in ascitic serum)	Glucose (5.0 m*M*)	60	0.088	0.188	0.388
L5178Y ascites	Nil	0	0.028	0.054	0.097
Lymphoma	Nil	60	0.021	0.028	0.297
(in ascitic serum)	Glucose (5.0 m*M*)	60	0.028	0.022	0.212
Ehrlich ascites	Nil	0	0.051	0.073	0.681
Carcinoma	Nil	60	0.033	0.046	0.551
(in buffer)	Glucose (5.0 m*M*)	60	0.028	0.031	0.596

alter this situation, except insofar as the presence of glucose or uridine in the incubation medium tends to depress the ATP level slightly (Tables II and III). Similar results are obtained if the cells are incubated, not in their own serum, but in Krebs–Ringer bicarbonate buffer (Table II). The situation during anaerobic incubation is quite different: within 15 min there is a sharp fall in ATP content, though this can be delayed by addition of glucose (Table III). Addition of uridine has no effect. The source of the energy which the cells obtain when incubated aerobically in the presence of glucose may be found, at least in part, in the oxidation of lactate and pyruvate, which are present in high concentration in the tumour *in vivo* and which are measurably diminished during aerobic incubation *in vitro* (Table IV).

It was previously shown that the incorporation of [8-^{14}C]adenine and [6-^{14}C]orotic acid into the nucleic acids of Ehrlich ascites tumour cells incubated *in vitro* under aerobic conditions is substantially increased by addition of glucose[4]. Table V shows that under such conditions glucose also enhances uptake of both these precursors and of [2-^{14}C]uracil from the ascitic serum into the cells. In contrast, uptake of [2-^{14}C]-glycine and [2-^{14}C]phenylalanine during aerobic incubation is unaffected by addition

TABLE III

EFFECT OF GLUCOSE, URIDINE AND IODOACETATE ON THE ATP CONTENT OF THE EHRLICH ASCITES CARCINOMA *in vitro*

Gas phase	*Time of incubation (min)*	*Additions*	*ATP content (µmole/ml)*
Air	0	Nil	0.414
	45	Nil	0.526
	45	Glucose (20 m*M*)	0.396
	45	Uridine (0.5 m*M*)	0.396
	45	Iodoacetate (1.0 m*M*)	0.084
N_2	0	Nil	0.368
	15	Nil	0.086
	40	Nil	0.073
	15	Glucose (10 m*M*)	0.465
	40	Glucose (10 m*M*)	0.110
	15	Uridine (0.5 m*M*)	0.060
	40	Uridine (0.5 m*M*)	0.048

of glucose. Under anaerobic conditions uptake of [2-^{14}C]phenylalanine is depressed, but can be restored by addition of glucose (Table V). Uptake of [2-^{14}C]uracil under aerobic conditions is increased by addition of adenosine; and, conversely, uptake of [8-^{14}C]adenine is increased by addition of uridine (Table V). When [8-^{14}C]adenine or [^{14}C]uracil is taken up by ascites cells *in vitro*, part of the radioactivity in the cells is found in the form of nucleotides and part as free base (Table VI). If either glucose

TABLE IV

EFFECT OF INCUBATION *in vitro* ON LACTATE AND PYRUVATE CONTENT OF THE EHRLICH ASCITES CARCINOMA

Time (min)	*Gas phase*	*μmoles/ml lactate*	*Tumour pyruvate*
0	—	10.9	0.23
40	Air	10.4	0.24
120	Air	9.0	0.08
40	O_2	10.2	—
120	O_2	9.9	—

TABLE V

THE EFFECT OF GLUCOSE, RIBOSE AND NUCLEOSIDES ON UPTAKE OF LABELED AMINO ACIDS, PURINES AND PYRIMIDINES BY EHRLICH ASCITES CARCINOMA CELLS *in vitro*

	Gas phase	*Control*	*Percentage of added radioactivity taken up by cells*				
			+ Glucose (5 mM)	*+ Glucose (20 mM)*	*Uridine (0.5 mM)*	*Adenosine (0.5 mM)*	*Ribose (20 mM)*
0.025 m*M* [8-^{14}C]adenine	Air	47	62	70	73	78	—
0.043 m*M* [2-^{14}C]uracil	Air	4	16	25	—	30	3
0.043 m*M* [6-^{14}C]orotic acid	Air	4	6	9	—	—	—
0.053 m*M* [2-^{14}C]glycine	Air	46	48	46	—	—	—
0.020 m*M* [2-^{14}C]phenylalanine	Air	40	43	44	—	—	—
Ditto	N_2	28	33	45	—	—	—

TABLE VI

DISTRIBUTION OF RADIOACTIVITY IN ACID-SOLUBLE FRACTION OF EHRLICH ASCITES CARCINOMA CELLS INCUBATED AEROBICALLY *in vitro*

Incubation in presence of 0.025 m*M* [8-^{14}C]adenine or 0.043 m*M* [2-^{14}C]uracil. Time of incubation, 60 min.

Form of isotope	*Additions to incubation medium*	*% total radioactivity in form of*		
		Free base	*Nucleoside*	*Nucleotides*
[8-^{14}C]adenine	Nil	26	0	71
	Glucose (5.0 m*M*)	4	0	96
	Adenosine (0.5 m*M*)	2	0	96
	Uridine (0.5 m*M*)	8	0	91
[2-^{14}C]uracil	Nil	86	0	14
	Glucose (2.0 m*M*)	20	0	80
	Adenosine (0.5 m*M*)	13	1	86
	Uridine (0.5 m*M*)	39	2	59

or a nucleoside is added to the incubation medium, the proportion in the nucleotide form is substantially increased.

Rabbit bone marrow

The behaviour of rabbit bone marrow differs radically from that of the Ehrlich ascites carcinoma. Glucose and pyrimidine nucleosides have little or no effect on incorporation of [^{14}C]formate into nucleic acid adenine *in vitro* (Table I). A mixture of glucose, glutamine, aspartic acid and glycine produces a slight increase in incorporation which is more easily demonstrated if the cells are carefully washed with buffer before the start of the experiment (Table I). But even under these conditions the specific activities of DNA and RNA adenine remain very low relative to those of DNA thymine. Boiled rat liver extract, which, like glucose, stimulates incorporation of [^{14}C]formate into the purine nucleotides of the Ehrlich ascites carcinoma *in vitro*[3] does not produce an analogous effect on bone marrow. Blood serum, which also has a specific stimulating effect on purine nucleotide biosynthesis in the Ehrlich ascites tumour *in vitro*[3], stimulates incorporation of [^{14}C]formate equally into nucleic acid purines and DNA thymine *in vitro* (Table I). Finally, the ability of the bone marrow cells to incorporate [^{14}C]formate into their nucleic acid bases *in vitro* is very little impaired if the cells are incubated in buffer for up to 60 min before adding the isotope (Table I). This is in marked contrast to the Ehrlich ascites carcinoma, which, when similarly treated, shows a marked reduction in its capacity to incorporate [^{14}C]formate (Table I).

Other tissues

Incorporation of [^{14}C]formate has also been investigated in two other neoplastic tissues, the L5178Y ascites lymphoma and a transplantable mouse mammary adenocarcinoma (Table VII). In contrast to the results obtained with the Ehrlich ascites carcinoma, the activity of the nucleic acid adenine in the ascites lymphoma is quite comparable with that of the DNA thymine (Table VII). The effect of glucose on the ascites lymphoma is to increase incorporation into nucleic acid adenine and DNA thymine to approximately the same extent (Table VII). There is no suggestion of a specific effect on purine nucleotide biosynthesis. Addition of uridine does not increase incorporation into nucleic acid adenine (Table VII). These differences between the two ascites tumours do not appear to be attributable to differences in the amount of energy available to them. The relative proportions of ATP, ADP and AMP are roughly the same in both, and are similarly affected by incubation in presence and absence of glucose (Table II). Examination of the two tumours, however, shows that *in vivo* the lymphoma has a glucose content which varies from animal to animal but which is generally in the range of 10 to 40 mg/100 ml tumour. The corresponding figure for the Ehrlich ascites carcinoma is always less than 3 mg/100 ml. This difference *in vivo* is reflected in a difference in the rate at which the cells of the two tumours take up added glucose *in vitro*. Fig. 1 shows that the rate of uptake is very much more rapid in the carcinoma than in the lymphoma. The differences in the glucose requirement of the two tumours may in turn reflect differences in their mode of growth. Although the density of the cell population is greater in the lymphoma—approximately $170 \cdot 10^6$ cells per ml compared to approximately $80 \cdot 10^6$ cells per ml in the carcinoma—the carcinoma gives a much higher packed cell volume when centrifuged in a haematocrit

TABLE VII

INCORPORATION OF [^{14}C]FORMATE INTO THE ACID-SOLUBLE AND NUCLEIC ACID BASES OF AVIAN AND MAMMALIAN TISSUES *in vitro*

Initial isotope concentration 5 μC/ml.

Tissue	*Additions to incubation medium*	*Specific activity (counts/min/μmole)*			
		Acid-soluble adenine	*RNA adenine*	*DNA adenine*	*DNA Thymine*
L5178Y ascites lymphoma	Nil	—	7,790	2,900	10,840
	Glucose (5.0 m*M*)	—	22,100	8,540	31,780
	Uridine (0.5 m*M*)	—	8,650	1,488	10,400
Solid mammary adenocarcinoma	Nil	—	61,250	6,600	11,280
	Glucose (5.0 m*M*)	—	194,100	28,390	13,070
Adult rat brain	Nil	7,000	440	—	—
	Glucose (5.0 m*M*)	22,200	3,480	—	—
Chick embryo brain	Nil	89,000	7,700	1,630	15,500
	Glucose (5.0 m*M*), glutamine (0.5 m*M*)	88,000	9,400	1,650	28,300
Chick embryo skeletal muscle	Nil	14,600	1,300	494	30,300
	Glucose (5.0 m*M*), glutamine (0.5 m*M*)	128,000	10,300	2,220	45,800
Solid Ehrlich carcinoma	Nil	—	15,150	700	3,970
	Glucose (5.0 m*M*)	—	60,900	3,730	18,730
Solid L5178Y lymphoma	Nil	—	1,360	420	2,360
	Glucose (5.0 m*M*)	—	6,940	1,860	14,400

tube, 26 to 32 % of the tumour cell suspension compared to 11 to 22 % for the lymphoma. On the average, therefore, the mass of cells per ml of tumour cell suspension is about twice as high in the carcinoma as it is in the lymphoma.

To determine whether the *in vitro* responses to glucose of the two ascites tumours are due to inherited cell characteristics or merely to their relationship with their environment *in vivo*, both tumours were grown subcutaneously in the solid form. When slices of such tumours are incubated *in vitro* with [^{14}C]formate both give similar incorporation patterns in which the activity of the DNA thymine is approximately five times that of the DNA adenine (Table VII). In both cases addition of glucose to

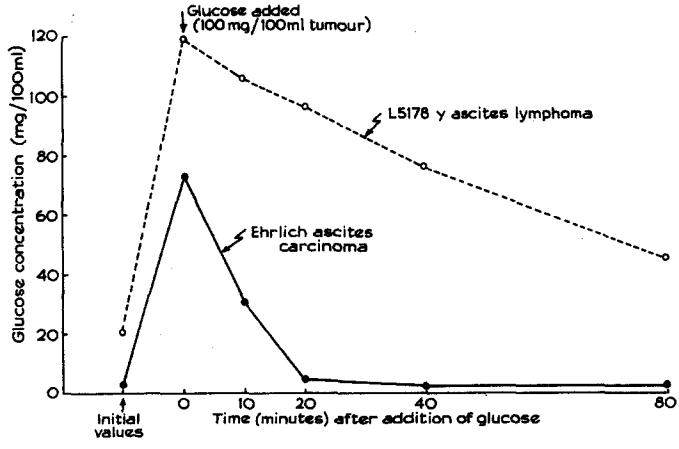

Fig. 1. Uptake of glucose by Ehrlich ascites carcinoma and L5178 Y ascites lymphoma *in vitro*.

the incubation medium increases incorporation into DNA thymine and DNA and RNA adenine to about the same extent (Table VIII).

The solid adenocarcinoma gives rather different results. *In vitro*, like the ascites lymphoma, it incorporates [^{14}C]formate extensively into nucleic acid adenine as well as into DNA thymine (Table VII). Glucose, however, produces a threefold increase in incorporation into nucleic acid adenine, without greatly affecting incorporation into DNA thymine. To determine whether other rapidly-growing normal tissues might behave in the same manner, the effects of glucose (supplemented with glutamine) on chick embryo brain and skeletal muscle was investigated (Table VII). In both cases the controls show much greater incorporation into DNA thymine than into nucleic acid adenine. Addition of glucose and glutamine increases the specific activity of DNA thymine by about 50 % in both. In skeletal muscle it also increases incorporation into acid-soluble and nucleic acid adenine 5- to 8-fold, but even under these circumstances the specific activity of the DNA adenine is still only about 5 % that of the DNA thymine. In embryo brain, on the other hand, glucose and glutamine appear to have no effect whatever on incorporation of [^{14}C]formate into acid-soluble and nucleic acid adenine. This is in sharp contrast to the situation in adult rat brain, where addition of glucose and glutamine more than trebles incorporation of [^{14}C]-formate into acid-soluble and RNA adenine *in vitro* (Table VIII).

TABLE VIII

DISTRIBUTION OF RADIOACTIVITY BETWEEN C-2 AND C-8 OF ACID-SOLUBLE ADENINE IN EHRLICH ASCITES CARCINOMA AND RABBIT BONE MARROW CELLS FOLLOWING INCUBATION FOR 4 H *in vitro* IN PRESENCE OF [^{14}C]FORMATE

	% *radioactivity*	
	C-2	*C-8*
Ehrlich ascites carcinoma	91	9
Rabbit bone marrow	61	39

DISCUSSION

The results described above go some way toward answering the three questions raised in the Introduction.

The influence of glucose on the Ehrlich ascites carcinoma

Two alternative hypotheses have been put forward to explain the fact that addition of glucose greatly increases purine biosynthesis in the Ehrlich ascites carcinoma *in vitro*.

1. HARRINGTON[6] has suggested that the glucose may act as a source of the ribose necessary for nucleotide biosynthesis. The principal evidence for this view has hitherto been the fact that low concentrations of cytidine and uridine (0.5 m*M*), which could liberate ribose-1-phosphate by the action of nucleoside phosphorylase, produce the same effect as much higher concentrations of glucose.

2. The validity of HARRINGTON's "ribose theory" has been challenged by HENDERSON AND LEPAGE[15], who have shown that the effects of glucose are abolished

by iodoacetate (alone or with dinitrophenol). From this they conclude that the "principal role of glucose is as an energy supply". The abolition of the glucose effect by iodoacetate is not reversed by methylene blue, which might be expected to increase oxidation of glucose and consequent production of pentose phosphate. This observation leads HENDERSON AND LEPAGE[15] to believe that the effect of glucose as a source of ribose is "a secondary consideration". The results of the present series of experiments support the first of these hypotheses rather than the second. It is quite clear from Tables II and III that when the Ehrlich ascites carcinoma is incubated aerobically *in vitro* the cells have a high content of ATP. Their failure to synthesize purines under these conditions cannot therefore be due to lack of the necessary energy. Moreover, under aerobic conditions glucose and uridine do not raise the ATP level, but tend rather to depress it. The increase in purine nucleotide biosynthesis which they produce cannot therefore be due to their acting as sources of energy. There remains the possibility that they are acting as sources of ribose.

Other evidence supports this opinion. The present experiments (Table III) have shown that when the tumour cells are incubated *in vitro* without glucose, their ATP content depends on the O_2 supply available. Under aerobic conditions it remains high, but under N_2 it falls off rapidly. In a previous paper[4] it was shown that *in vitro* increasing the amount of O_2 available to the cells also increased nucleic acid biosynthesis as indicated by incorporation of a wide range of precursors. This increase is presumably due to the effect of O_2 in maintaining the ATP content of the cells at a high level. Addition of glucose, on the other hand, which does not increase the ATP level of cells under aerobic conditions (Tables II and III), has little effect on nucleic acid biosynthesis (as indicated by incorporation of $^{32}PO_4$ (see ref. 4)). It does, however, produce a great increase in purine nucleotide biosynthesis as indicated by [^{14}C]formate incorporation (Table I). It thus appears that oxygen supply determines the energy available to the cells and therefore the rate at which they synthesize nucleic acids; but that, even under aerobic conditions when the content of ATP in the cells is high, an external supply of glucose is needed for purine nucleotide biosynthesis. Under these circumstances the glucose is presumably required as a source, not of energy, but of ribose phosphate.

This conclusion is not inconsistent with the observation of HENDERSON AND LEPAGE[15] that iodoacetate abolishes the stimulatory effect of glucose on incorporation of [2-^{14}C]glycine into the acid-soluble adenine of the cells. Iodoacetate is not a highly specific enzyme inhibitor and even in absence of added glucose it depresses the ATP level of the cells during aerobic incubation (Table III). It has been shown above that under such conditions the cells contain no free glucose, but appear to maintain their ATP content by oxidative phosphorylation, probably at the expense of lactate and pyruvate. Iodoacetate presumably must inhibit some stage of this process. Its action therefore, in reversing the stimulating effects of glucose, does not prove that these effects are a result of glycolysis. It probably only reflects the fact that purine nucleotide biosynthesis requires ATP as well as ribose phosphate, without indicating the sources from which the ATP is derived. If this is so, it is not surprising that, as HENDERSON AND LEPAGE[15] have shown, the use of methylene blue (to increase glucose oxidation and hence the supply of ribose) fails to reverse the inhibitory effects of iodoacetate.

The theory that *in vitro* the external glucose supply determines the amount of ribose available for nucleotide biosynthesis in the Ehrlich ascites carcinoma is

supported by the results in Tables V and VI. These show that the degree to which uracil, adenine and orotic acid are taken up by the cells *in vitro* under aerobic conditions is increased in presence of glucose or nucleosides, and that the increase is accompanied by increased conversion of the free bases to the corresponding nucleotides. Since under these conditions the cells have a high ATP content whether glucose and nucleosides are present or not (Tables II and III) the effects produced by these compounds are presumably due to their acting as sources, not of energy, but of the ribose phosphate necessary for nucleotide synthesis. Free ribose is ineffective, just as it is ineffective in stimulating purine biosynthesis *in vitro*[6]. Presumably the cells lack the ribokinase necessary to phosphorylate it[6]. In contrast, the uptake of glycine and phenylalanine under aerobic conditions is unaffected by addition of glucose (Table V). Under aerobic conditions, when the energy derived from oxidative phosphorylation is not available, uptake of phenylalanine is depressed. It can, however, be restored by addition of glucose, presumably because the latter can provide the necessary energy as a result of glycolysis (Table V). Uptake of phenylalanine, therefore, seems to depend on the energy available; uptake of adenine, uracil and orotic acid and their conversion to nucleotides depend also on the available supply of ribose phosphate.

Purine biosynthesis in rabbit bone marrow

At least two possible theories can be put forward to account for the very slow rate of purine nucleotide biosynthesis in rabbit bone marrow *in vitro*.

1. It is possible that *in vivo* the bone marrow might be supplied by the bloodstream with some precursor necessary for the synthesis (just as the Ehrlich ascites carcinoma is supplied with glucose); and that *in vitro* the absence of this precursor might diminish the rate of synthesis.

2. Alternatively, it is possible that the bone marrow cells may have only a very limited capacity for purine nucleotide biosynthesis and that *in vivo* they may obtain most of the purine they require from the bloodstream[16].

The present experiments give no support to the first of these hypotheses. Of the numerous precursors required for purine nucleotide biosynthesis[17], only glucose, glutamine, aspartic acid and glycine are likely to be present in the blood plasma, and it has been shown above that a mixture of all four has only a slight effect on incorporation of [^{14}C]formate into the purine nucleotides *in vitro* (Table I). Moreover, if purine biosynthesis in bone marrow *in vivo* depends on a supply of some precursor from the blood, addition of blood serum should increase purine biosynthesis in bone marrow cells *in vitro*. Table I, however, shows that blood serum has no specific effect on incorporation of [^{14}C]formate into nucleic acid purines, which is increased only to the same extent as incorporation into DNA thymine. Blood serum therefore appears, as THOMAS has already shown[18], to contain factors which stimulate biosynthesis of nucleic acids but not of purine nucleotides. This is in contrast to the Ehrlich ascites carcinoma, in which purine nucleotide biosynthesis is specifically increased by small amounts of blood serum[3], presumably because of its glucose content. Moreover, boiled liver extract, which also stimulates purine biosynthesis in the ascites tumour[3], and which might be expected to contain a wide variety of purine precursors, has no effect on bone marrow.

The second theory is more consistent with the marked differences in purine biosynthesis *in vitro* between bone marrow and the Ehrlich ascites carcinoma found in

the present experiments. Thus the very limited incorporation of [^{14}C]formate into acid-soluble purines in the Ehrlich ascites tumour *in vitro* in absence of glucose is confined chiefly to the first 30 min of incubation (Fig. 2). If addition of the isotope is delayed beyond this period, incorporation is greatly diminished (Table I). Even if there is no delay in addition of the isotope, incorporation is confined chiefly to the C-2 position (Table VIII). Since relatively little activity is found in C-8, it is probable that incorporation may be due chiefly to conversion of existing 5-amino-4-iminazolecarboxamide ribonucleotide to purine nucleotides (or to labeling of existing purine nucleotides by transformylation[15]) rather than to *de novo* purine nucleotide biosynthesis from glycine and phosphoribosyl pyrophosphate. This conclusion is supported by the relatively small decrease in incorporation brought about by azaserine, which is known to inhibit one of the early stages of purine nucleotide biosynthesis (Table IX). These observations suggest that in this tissue [^{14}C]formate incorporation into purines *in vitro* represents the last stage of a process of purine biosynthesis which is being brought to a standstill by lack of one of the precursors (glucose) necessary for its continuance.

In bone marrow, on the other hand, incorporation of [^{14}C]formate into acid-soluble purines, although slow, continues steadily for up to 4 h (Fig. 2), and the isotope incorporated is fairly equally distributed between the C-2 and C-8 positions of the purine nucleus (Table IX). Incorporation seems therefore to represent biosynthesis of purines *de novo* from glycine and phospho-ribosylamine, rather than from such intermediates as 5-amino-4-iminazolecarboxamide ribonucleotide. In agreement with this hypothesis, azaserine has a greater inhibitory effect on incorporation in bone marrow than in the Ehrlich ascites tumour (Table IX). Again in contrast to the ascites tumour, incorporation is not greatly diminished if addition of the isotope is delayed until, say,

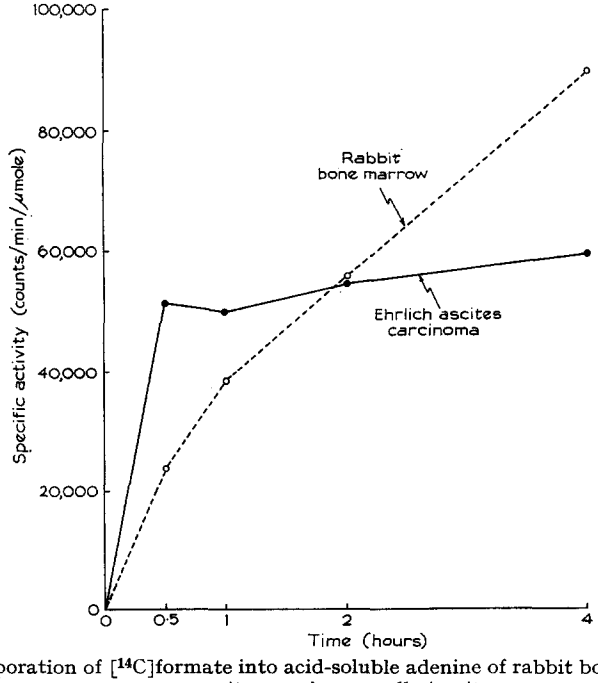

Fig. 2. Incorporation of [^{14}C]formate into acid-soluble adenine of rabbit bone marrow and Ehrlich ascites carcinoma cells *in vitro*.

TABLE IX

EFFECT OF AZASERINE ON INCORPORATION OF [^{14}C]FORMATE INTO THE NUCLEIC ACID BASES OF MAMMALIAN TISSUES *in vitro*

Results are expressed as percentages of control values.

	RNA adenine	*DNA adenine*	*DNA thymine*
Ehrlich ascites carcinoma	50	60	86
Rabbit bone marrow	20	19	89
Solid mammary adenocarcinoma	2	4	85
L5178Y ascites lymphoma	11	5	100

1 h after the beginning of the incubation (Table I). It therefore appears that removal of bone marrow from the animal and its subsequent incubation *in vitro* may not produce any very drastic change in its capacity for purine nucleotide biosynthesis. If this is so, bone marrow *in vivo* must synthesize purines relatively slowly and must depend largely on a supply of preformed purines from the blood.

Purine biosynthesis in other tissues

It has previously been shown that a low rate of purine biosynthesis *in vitro* (in absence of glucose) is found, not only in rabbit bone marrow and the Ehrlich ascites carcinoma, but also in a wide range of rapidly growing tissues[3]. The results in Table VII, however, show that this is not an invariable characteristic of such tissues. Thus the L5178Y ascites lymphoma, which *in vivo* grows at approximately the same rate as the Ehrlich ascites carcinoma, appears *in vitro* to synthesize purines much more rapidly (Tables I and VII). On the evidence presented above, this difference can be explained entirely in terms of glucose requirement. Both tumours can synthesize purines rapidly *in vitro* if provided with glucose. *In vivo*, however, the ascites lymphoma makes smaller demands on the supply of glucose available in the peritoneal cavity. Consequently it has a reserve of glucose, whereas the ascites carcinoma has none. Hence removal from the body of the host, with consequent cessation of glucose supply, has an inhibitory effect on purine biosynthesis in the carcinoma, but not in the lymphoma. When the two ascites tumours are grown in solid form, their fundamental similarity is strikingly illustrated. Slices of such tumours incubated *in vitro* in presence of [^{14}C]formate give essentially the same incorporation pattern (Table VII), in which the specific activity of the DNA adenine is about 20 % that of the DNA thymine. The responses of the two solid tumours to added glucose are exactly similar. Incorporation into both DNA thymine and nucleic acid adenine is increased approximately 4- to 6-fold (Table VIII). There is no indication of a specific effect on purine biosynthesis.

The solid mammary adenocarcinoma fits into the same pattern as the two ascites tumours since, if supplied with additional glucose, it can incorporate [^{14}C]formate extensively into nucleic acid adenine *in vitro* (Table VII). It may indeed be regarded as intermediate between them, resembling the lymphoma in its ability to synthesize purines in absence of any addition, and resembling the carcinoma in responding to glucose by specifically increasing this incorporation. Such a relationship between glucose supply and purine biosynthesis is not limited to neoplastic tissues. Table VIII shows that purine biosynthesis *in vitro* in adult rat brain, a tissue with small reserves

of glucose or glycogen, is substantially increased by addition of glucose and glutamine to the incubation medium. The behaviour *in vitro* of the chick embryo tissues, on the other hand, resembles that of bone marrow in that, even if they are supplemented with glucose and glutamine, their incorporation of [^{14}C]formate into DNA adenine is much lower than incorporation into DNA thymine (Table VIII). The presence of glucose and glutamine does not affect purine biosynthesis in chick embryo brain; in chick embryo muscle it produces some increase.

In conclusion, therefore, it appears that the tissues investigated so far fall into two main classes.

1. Those which, at least when supplied with glucose, are capable of incorporating [^{14}C]formate *in vitro* into DNA adenine and DNA thymine to approximately equal degrees (*i.e.* the Ehrlich and L5178Y tumours in both solid and ascites forms, the solid mammary adenocarcinoma, chick embryo liver and regenerating rat liver[3]).

2. Those which, even if supplied with purine precursors, do not incorporate [^{14}C]formate *in vitro* as extensively into DNA adenine as into DNA thymine (*i.e.* rabbit bone marrow and chick embryo brain and muscle).

Both classes include examples of tissues in which purine biosynthesis *in vitro* is increased in presence of glucose. This phenomenon therefore does not appear to be related to the ability of a tissue to synthesize for itself all the purine nucleotide which it requires. It seems rather to reflect the degree to which *in vivo* it depends on a continuous supply of glucose.

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