Purine biosynthesis *de novo* in rat skeletal muscle

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Purine biosynthesis by the '*de novo*' pathway was demonstrated in isolated rat extensor digitorum longus muscle with [1-¹⁴C]glycine, [3-¹⁴C]serine and sodium [¹⁴C]formate as nucleotide precursors. Evidence is presented which suggests that the source of glycine and serine for purine biosynthesis is extracellular rather than intracellular. The relative incorporation rates of the three precursors were formate > glycine > serine. Over 85% of the label from formate and glycine was recovered in the adenine nucleotides, principally ATP. Azaserine markedly inhibited purine biosynthesis from both formate and glycine. Cycloserine inhibited synthesis from serine, but not from formate. Adenine, hypoxanthine and adenosine markedly inhibited purine synthesis from sodium [¹⁴C]formate.

Purine nucleotides can be synthesized by two pathways, the '*de novo*' pathway, which predominantly uses amino acids, and the salvage pathway, which uses preformed purine bases and nucleosides. Tissues appear to differ in their capacities to synthesize nucleotides by one or both of these pathways.

Murray (1971) commented that there was no information on whether muscle tissue can synthesize purines *de novo* or whether depleted nucleotide pools must be replenished by purine salvage. McKeran & Watts (1978) noted that the importance in muscle of purine biosynthesis *de novo* remained to be established. In preliminary reports, evidence for the ability of rat extensor digitorum longus muscle to synthesize purine nucleotides by the '*de novo*' pathway was presented (Sheehan *et al.*, 1977; Tully & Sheehan, 1980). A full account of this work is presented here.

Work with primary rat muscle cell cultures supports the conclusion that skeletal muscle utilizes the '*de novo*' pathway for purine nucleotide synthesis (Brosh *et al.*, 1982; Zoref-Shani *et al.*, 1982). Interest in the study of muscle purine metabolism has been stimulated by the suggestion that allopurinol, a xanthine oxidase inhibitor, improves patients with Duchenne muscular dystrophy (Thomson & Smith, 1978), and also by the report of a muscle adenylate deaminase deficiency associated with skeletal-muscle dysfunction (Fishbein *et al.*, 1978).

Materials and methods

Materials

Isotopically labelled compounds were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Polygram Cel 300 poly(ethyleneimine)-cellulose thin-layer plates were from Macherey, Nagel and Co., Düren, Germany. Chromagram cellulose thin-layer plates were obtained from Eastman Kodak Co., Rochester, NY, U.S.A., and DEAE-cellulose paper was from W. and R. Balston, Maidstone, Kent, U.K. Albino rats (70–90 g) of the Wistar strain obtained from the University animal house were used. The animals were fed on standard rat pellets (Livestock Foods Ltd., Dublin, Ireland), and were allowed food *ad libitum*. Both male and female rats were used, as sex differences did not affect the results obtained.

Isolated muscle preparation

Rat extensor digitorum longus muscle was used. The animals were anaesthetized with pentobarbitone sodium (50 mg/kg), and the muscles, weighing between 25 and 35 mg, were removed. Muscles of this size were selected since the oxygen uptake during incubation becomes critical if larger muscles are used (Fern *et al.*, 1971). Both the dry and wet muscle weights were directly proportional to the weight of the whole rat. The relationship

$$\text{Rat weight (g)} = 2.3 \text{ muscle weight (mg)} + 9$$

gave a correlation coefficient of 0.92 for 25 rats. This avoided the necessity for direct weighing of the

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muscle. Owing to its small size, four muscles were used in each incubation, so that changes in metabolite concentration could be measured more accurately. Details of the incubation are given by Sheehan *et al.* (1977).

Extraction of purines

Muscles were removed from the incubation medium, quickly rinsed in cold physiological saline (0.9% NaCl), and freeze-clamped with a clamp pre-cooled in liquid N₂. The frozen tissue was placed in a plastic centrifuge tube filled with liquid N₂ and ground to a powder with a round-ended steel rod. The residual liquid N₂ was allowed to evaporate. After addition of 0.5 ml of 0.92 M-HClO₄, the suspension was homogenized for 15 s with an Ultra-Turrax TP 18/10 blender, and centrifuged at 4000 g for 5 min. The suspension was then centrifuged at 4000 g for 5 min at 4°C, and the supernatant neutralized with cold KOH, saturated with KCl. KCl saturation ensured that KClO₄ concentration was minimized by the common-ion effect (Buckley & Williamson, 1977).

The neutralized extract was left at 0°C for 20 min to allow full precipitation of the KClO₄, after which it was centrifuged at 1500 g for 5 min at 4°C. The extract was analysed immediately after preparation.

Separation of purine nucleotides

Purine nucleotides were separated by t.l.c. on poly(ethyleneimine)-cellulose plates (Henderson *et al.*, 1974). When labelled formate was used, it co-chromatographed with several nucleotides. The method of Mehra (1977) was used to separate the formate from these nucleotides. This method is essentially that of Neuhaard & Munch-Petersen (1966) with minor modifications. Purine nucleotide carrier mixture (5 µl) was spotted on a poly(ethyleneimine)-cellulose plate, followed by 20 µl of muscle extract. The plate was washed for 15 min in 500 ml of methanol to remove any interfering salt in the extract, and then air-dried. Development in the first dimension was with 1 M-LiCl/1 M-acetic acid (1:1, v/v). After air-drying, the plate was washed for 15 min in 500 ml of methanol, re-dried, and developed in the second dimension with 3.0 M-ammonium acetate/5% (w/v) boric acid (adjusted to pH 7.0 with ammonia).

Separation of purine bases and nucleosides

Bases and nucleosides were separated by two-dimensional chromatography on cellulose thin-layer sheets (Burrige *et al.*, 1977).

Detection and quantification of labelled purines after chromatography

After chromatography, purine spots were located under u.v. light, and circled with a pin. Nucleotide

spots were moistened with 1.5 M-NaCl with a 10 µl Drummond 'microcap' pipette, removed from the plastic backing with a spatula, and transferred quantitatively to vials for radioactivity counting. Purine bases and nucleosides were treated similarly, except that the spots were moistened with 0.05 M-Tris/HCl, pH 7.0. The composition of the scintillation fluid was 5.0 g of 2,5-diphenyloxazole, 0.5 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 330 ml of Triton X-100 made up to 1 litre with toluene. The radioactivity was measured in a Beckman LS-100C liquid-scintillation counter.

Measurement of amino acids in muscle and incubation medium

Analysis was performed by a modification of the g.l.c. method of Gehrke & Leimer (1971), which consisted of adjusting the initial temperature, holding time and temperature-gradient rate, to decrease the separation time from 42 to 18 min. Interfering substances were removed by the method of Zumwalt *et al.* (1970). The amino acid trimethylsilyl derivatives were separated in a Perkin-Elmer Sigma 3B temperature-programmed gas chromatograph, equipped with a two-column oven and two flame ionization detectors.

Results

Viability of muscle preparation

In a preliminary report, evidence was presented that the isolated extensor digitorum longus muscle remained viable over a 60 min incubation period (Sheehan *et al.*, 1977). The criteria employed were glucose uptake, lactate release and intracellular concentrations of AMP, ADP, ATP and K⁺. As experiments involving incubation periods of up to 3 h were performed, viability studies were extended over that period. In addition to the previous criteria used, phosphocreatine concentrations, muscle weight, water content and extracellular space were determined. All criteria indicated a viable muscle preparation over the 3 h incubation period.

Purine biosynthesis from labelled precursors

Purine biosynthesis by the 'de novo' pathway was demonstrated by the use of [1-¹⁴C]glycine and [3-¹⁴C]serine as precursor molecules (Fig. 1). The extensor digitorum longus muscle excreted glycine and serine into the incubation medium, so the specific radioactivities of those amino acids both intracellularly and in the medium were monitored over the incubation period. The values obtained were used to calculate the mean specific radioactivities of both amino acids in the muscle and medium (Zimmer *et al.*, 1973). The mean specific radioactivities were then employed to calculate the rates of purine synthesis from the two amino acids. The

Fig. 1. Purine synthesis in extensor digitorum longus muscle from amino acid precursors

Muscles were incubated at 37°C in physiological saline (Krebs & Henseleit, 1932) containing 5 mM-glucose, 1.5 g of bovine serum albumin/l, 0.2% silicone antifoam emulsion and either 0.3 mM-[1-¹⁴C]glycine (Δ) or 0.3 mM-[3-¹⁴C]serine (□). The medium was bubbled throughout with O₂/CO₂ (19:1). Muscles were kept at resting length under 2 g tension. Labelled purines were extracted, separated and measured as detailed in the Materials and methods section. Values shown are the radioactivities incorporated into each total purine fraction and are the averages of two experiments.

results are shown in Tables 1 and 2. Fig. 1 shows that glycine was incorporated into purines at a linear rate for the first 60 min. Purine labelling was decreased by approx. 60% between 60 and 120 min, and no net incorporation of label occurred after 120 min.

When [¹⁴C]serine was used as precursor, the rate of labelling proceeded in a sigmoidal fashion over the 3 h period. The slow rate in the first 60 min may be due to the fact that serine is not directly incorporated, but must first donate its labelled carbon to a folate coenzyme.

In Table 1 the rates of synthesis *de novo* from glycine have been calculated from the mean specific-radioactivity values of both intracellular and extracellular glycine. The rate calculated from intracellular values is very high, and falls markedly over the first 60 min. Values of this magnitude have not been reported for other animal tissues. The results suggest that the intracellular amino acid pools are not uniformly labelled.

When sodium [¹⁴C]formate was used to study purine synthesis *de novo*, it was assumed that intracellular formate concentration was negligible, as few biological sources of formate are known (Henderson, 1972). Preliminary experiments showed

Table 1. Rates of purine biosynthesis *de novo* in extensor digitorum longus muscle from [1-¹⁴C]glycine

Rates were calculated by dividing the amount of label incorporated into purines by the corresponding mean specific radioactivities of glycine. Rate (A) was calculated from the mean specific radioactivities of intracellular glycine, and rate (B) from those of extracellular glycine.

Incubation period (min)	Mean sp. radioactivity of glycine (c.p.m./nmol)		Rates of synthesis <i>de novo</i> (nmol/h per g)	
	(A)	(B)	(A)	(B)
	Intracellular	Extracellular		
0-5	335	62 000	2190	12
5-15	1990	61 000	480	14
15-30	3360	60 000	202	13
30-45	4710	58 700	136	11
45-60	5590	57 500	93	10
60-90	6040	52 800	56	7
90-120	6150	48 900	23	3
120-180	6340	45 100	3	0.4

Table 2. Rates of purine biosynthesis *de novo* in extensor digitorum longus muscle from [3-¹⁴C]serine

Rates were calculated by dividing the amount of label incorporated into purines by the corresponding mean specific radioactivities of serine. Rate (A) was calculated from the mean specific radioactivities of intracellular serine, and rate (B) from those of extracellular serine.

Incubation (min)	Mean sp. radioactivity of serine (c.p.m./nmol)		Rates of synthesis <i>de novo</i> (nmol/h per g)	
	(A)	(B)	(A)	(B)
	Intracellular	Extracellular		
0-5	300	60 100	100	0.5
5-15	1320	58 200	73	1.7
15-30	3300	56 800	68	4.0
30-45	5400	56 900	56	5.3
45-60	7250	57 000	63	8.1
60-90	9490	56 700	49	8.1
90-120	10 700	55 800	15	2.9
120-180	11 000	53 600	7	1.4

that formate entered muscle and that 10% was converted into purine compounds, the remainder being unmetabolized. Labelling of purines was linear over the first 60 min, but fell over the next 2 h. Assuming that the intracellular and extracellular specific radioactivities were identical, the rate of purine synthesis for the first 1 h of incubation was 28.1 ± 1.1 nmol/h per g (mean $\pm$ s.d., $n = 4$).

Since formate and serine may compete to supply C₁ units to tetrahydrofolate, the effect of unlabelled

Table 3. *Incorporation of radioactivity into muscle purines from [1-¹⁴C]glycine and sodium [¹⁴C]formate*

Extensor digitorum longus muscle was incubated with 0.3 mM-[1-¹⁴C]glycine or 1 mM-sodium [¹⁴C]formate for 60 min. Labelled purines were extracted, separated and measured, as detailed in the Materials and methods section. The relative distribution values are expressed as percentages ($\pm$ S.D.) of the total radioactivity of all purine compounds in both muscle and incubation medium. These values are the means $\pm$ S.D. for four experiments.

Purine labelled	(a) Label incorporation (c.p.m./h per 20 μ l of muscle extract)		(b) Relative distribution of label between purines	
	Glycine	Formate	Glycine	Formate
ATP	7310 $\pm$ 576	34 900 $\pm$ 988	77.4 $\pm$ 6.1	76.8 $\pm$ 2.2
ADP	628 $\pm$ 86	3460 $\pm$ 542	6.6 $\pm$ 0.8	7.6 $\pm$ 1.2
AMP	179 $\pm$ 38	500 $\pm$ 138	1.9 $\pm$ 0.4	1.1 $\pm$ 0.3
NAD ⁺	132 $\pm$ 37	364 $\pm$ 93	1.4 $\pm$ 0.4	0.8 $\pm$ 0.2
GTP	567 $\pm$ 48	3180 $\pm$ 546	5.9 $\pm$ 0.5	7.0 $\pm$ 1.2
GDP	151 $\pm$ 38	637 $\pm$ 138	1.6 $\pm$ 0.4	1.4 $\pm$ 0.3
GMP	132 $\pm$ 47	409 $\pm$ 182	1.4 $\pm$ 0.5	0.9 $\pm$ 0.4
IMP	170 $\pm$ 48	1090 $\pm$ 48	1.8 $\pm$ 0.5	2.4 $\pm$ 0.1
XMP	123 $\pm$ 38	682 $\pm$ 227	1.3 $\pm$ 0.4	1.5 $\pm$ 0.5
Inosine	38 $\pm$ 29	136 $\pm$ 92	0.4 $\pm$ 0.3	0.3 $\pm$ 0.2
Hypoxanthine	28 $\pm$ 26	91 $\pm$ 90	0.3 $\pm$ 0.3	0.2 $\pm$ 0.2

Table 4. *Inhibition of purine biosynthesis de novo by azaserine*

Extensor digitorum longus muscle was preincubated for 15 min with azaserine or azaserine plus glutamine. Sodium [¹⁴C]formate (1 mM) or [1-¹⁴C]glycine (0.3 mM) was added and incubation continued for a further 60 min. Rates of synthesis *de novo* were calculated as detailed above. ATP concentrations were determined by the method of Lamprecht & Trautschold (1974). Values are means $\pm$ S.D. for the numbers of experiments shown in parentheses; N.D., not determined.

Precursor	Azaserine (mM)	Rates of <i>de novo</i> synthesis (nmol/h per g)	Effect (%)	ATP (μ mol/g of muscle)
[¹⁴ C]Formate	0	28.0 $\pm$ 1.1 (4)	0	4.94 $\pm$ 0.5 (4)
[¹⁴ C]Formate	0.01	16.1 $\pm$ 0.7 (4)	-43	4.70 (2)
[¹⁴ C]Formate	0.1	8.2 $\pm$ 1.3 (3)	-71	5.11 (2)
[¹⁴ C]Formate	1.0	3.4 $\pm$ 1.0 (3)	-88	5.06 (2)
[¹⁴ C]Formate	10	0.9 $\pm$ 0.4 (4)	-97	4.20 $\pm$ 0.3 (4)
[¹⁴ C]Formate plus 0.1 M-glutamine	10	5.2 $\pm$ 0.4 (3)	-82	4.79 $\pm$ 0.5 (3)
[¹⁴ C]Formate plus 0.1 M-glutamine	0	30.2 $\pm$ 2.7 (4)	+8 (stimulation)	4.82 $\pm$ 0.7 (4)
[1- ¹⁴ C]Glycine	0	11.9 $\pm$ 1.4 (5)	0	N.D.
[1- ¹⁴ C]Glycine	1	0.8 $\pm$ 0.2 (3)	-93	N.D.

formate on [3-¹⁴C]serine incorporation into muscle purines was investigated. Formate (1 mM) inhibited serine incorporation by 25%.

Purine labelling from [1-¹⁴C]glycine and sodium [¹⁴C]formate

Incorporation of label into individual purines is shown in Table 3(a), which demonstrates that over 85% of the label is recovered in the adenine nucleotides, predominantly in ATP. Labelling of guanine nucleotides was approx. 10% that of adenine nucleotides. The relative distribution of label between purines from glycine and formate is shown in Table 3(b). No significant difference was found, indicating that both precursors label a common pathway of purine synthesis in muscle.

Effects of azaserine and cycloserine on incorporation of labelled precursors of the 'de novo' pathway

Azaserine (O-diazoacetyl-L-serine) inhibits purine biosynthesis by the '*de novo*' pathway, acting as a classical competitive inhibitor because of its structural resemblance to glutamine (Henderson & Paterson, 1973). Azaserine markedly inhibited labelling of purines by both glycine and formate. The inhibition was decreased in the presence of glutamine (Table 4).

At 5 mM, cycloserine (D-4-amino-3-isoxazolidinone), which inhibits serine hydroxymethyltransferase, decreased labelling of purine nucleotides from [3-¹⁴C]serine by 40%, but had no effect on purine

Table 5. *Effects of exogenous adenine, hypoxanthine and adenosine on the rate of purine biosynthesis de novo from sodium [^{14}C]formate*

Extensor digitorum longus muscle was incubated for 60 min in a medium containing 0.1 mM-sodium [^{14}C]formate and hypoxanthine, adenine or adenosine at the indicated concentrations. Values represent means $\pm$ S.D. for four experiments.

Purine added	Concn. (μM)	Rates of synthesis <i>de novo</i>	
		(nmol/h per g)	(% of control)
None	—	13.0 $\pm$ 2.1	100
Hypoxanthine	20	3.4 $\pm$ 0.7	26
	1000	2.9 $\pm$ 0.4	22
Adenine	20	1.7 $\pm$ 0.2	13
	1000	1.4 $\pm$ 0.4	11
Adenosine	20	13.2 $\pm$ 2.7	102
	1000	6.9 $\pm$ 1.4	53

labelling from sodium [^{14}C]formate (results not shown). The inhibitor had no effect on serine uptake by muscle.

Effects of adenine, hypoxanthine and adenosine on purine synthesis from sodium [^{14}C]formate

Table 5 shows the effects of these purines on sodium [^{14}C]formate incorporation into purines: with the exception of adenosine at the lower concentration, all purines showed marked inhibition.

Discussion

Brosh *et al.* (1982) reported that sodium [^{14}C]formate was incorporated into purines by both myoblast and primary muscle cultures from the rat. The same authors also demonstrated synthesis *de novo* of purine nucleotides in muscles of mice injected with sodium [^{14}C]formate. Zoref-Shani *et al.* (1982) also provided evidence for purine nucleotide synthesis in primary rat muscle cultures, and showed that the rate of formate incorporation was dependent upon cellular availability of 5-phosphoribosyl 1-pyrophosphate.

The results presented here show clearly that the isolated extensor digitorum longus muscle of the rat can synthesize purine nucleotides by the '*de novo*' pathway. Partial supporting evidence was presented previously (Sheehan *et al.*, 1977).

Three precursors of purine nucleotide synthesis by the '*de novo*' pathway, glycine, serine and formate, were shown to be incorporated into the purines of the extensor digitorum longus muscle (Fig. 1 and Table 3). Table 1 shows that the rate of purine synthesis from glycine fell dramatically over a 60 min incubation when the mean specific radio-

activity of intracellular glycine was used in the calculation, but this is at variance with the linear rate of glycine incorporation over 60 min shown in Fig. 1. The rates calculated from the mean specific-radioactivity values of extracellular glycine are more in agreement. Other workers have failed to observe a direct relationship between the intracellular glycine pool and the rate of protein synthesis (Kipnis *et al.*, 1961; Manchester & Wool, 1963; Rosenberg & Holmsen, 1968; Roscoe *et al.*, 1968). Banos *et al.* (1973) reported that the entry of amino acids into rat skeletal muscle takes place by carrier-mediated transport processes with a high degree of specificity. They proposed a model for amino acid utilization for protein synthesis which, if applied to purine nucleotide synthesis, would explain how extracellular glycine could be preferentially incorporated into purines. In this model the amino acid carrier has two possible routes. The first is to enter the intracellular pool, followed by dissociation to the free amino acid and carrier. The second involves conjugation with ATP or some other molecule, with subsequent removal for protein synthesis. The results obtained with serine also suggest that extracellular serine is used for purine synthesis in muscle (Fig. 1 and Table 2).

The approximate rate for the three precursors over 60 min varied from 8 nmol/g for serine to 10 nmol/g for glycine to 28 nmol/g for formate. Detailed studies of the rates of purine nucleotide synthesis in skeletal muscle have not been made, but Zimmer *et al.* (1973) reported a rate of 8.4 ± 1.42 nmol/h per g for the rate of synthesis of adenine nucleotides *de novo* in rat heart *in situ*.

Table 3 shows that use of either labelled glycine or formate as precursors of purine synthesis resulted in approx. 85% of the label being recovered in adenine-containing compounds. A similar pattern was obtained in cultured human fibroblasts by Zoref & Sperling (1980) and by Zoref *et al.* (1978). Surprisingly, using rat muscle cultures Zoref-Shani *et al.* (1982) found that over 50% of labelled formate was recovered by hypoxanthine-containing compounds. The higher recovery of labelled formate in hypoxanthine-containing compounds in muscle cultures reflects either a decreased incorporation into the adenine and guanine nucleotides, or a higher rate of catabolism of the adenine nucleotides in this preparation, which may render it a less adequate physiological model.

Table 4 shows that azaserine inhibited the utilization of labelled formate and glycine for purine synthesis. The dose required for 90% inhibition was greater than that required for 90% inhibition in mouse liver, kidney, lung, intestine and spleen (Moore & Le Page, 1957). The greater tolerance of skeletal muscle to azaserine could be due to the high concentration in this tissue of glutamine (Garber *et*

al., 1976), which has a protective effect against azaserine (Levenberg *et al.*, 1957; Chu & Henderson, 1972). The addition of glutamine with azaserine modified the inhibition, although glutamine alone did not stimulate purine biosynthesis *de novo* in extensor digitorum longus, unlike in some cells, where it is reported to be rate limiting (Henderson, 1962; Fontenelle & Henderson, 1969). Table 4 shows that the lower concentration of azaserine used did not affect the concentration of ATP, but 10mM-azaserine decreased ATP by 15%. Marchetti *et al.* (1970) found no change in rat liver ATP concentrations after treatment with azaserine, but Hyams *et al.* (1967) reported a 37% decrease in ATP with a higher azaserine concentration.

Cycloserine, an inhibitor of serine hydroxymethyltransferase, lowered the rate of purine biosynthesis *de novo* from [3-¹⁴C]serine, but did not affect the incorporation of sodium [¹⁴C]formate (Table 4).

Adenine, adenosine and hypoxanthine inhibited purine biosynthesis *de novo* (Table 5). Similar results were reported by Zoref-Shani *et al.* (1982) with primary rat muscle cultures from extensor digitorum longus, by Gordon *et al.* (1980) with human fibroblasts, by Hershfield & Seegmiller (1976) with human lymphoblasts and by Badenoch-Jones & Buttery (1976) with chicken liver and kidney. At the concentrations used, none of the purines affected the relative proportion of IMP converted into adenine and guanine nucleotides (results not shown). Hershfield & Seegmiller (1976), using concentrations of adenine that inhibited the '*de novo*' pathway by less than 40% in human lymphoblasts, found that adenine selectively inhibited adenine nucleotide synthesis, while stimulating guanine nucleotide synthesis to a lesser extent.

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