Inosine metabolism in the rat

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1. Uptake and subsequent metabolism of purine and ribose moieties was monitored after intravenous administration of doubly labelled inosine. 2. More than 95% was cleared from the plasma within 5 min, and 99% within 20 min. 3. Approx. 50% of the 160 μ mol total was rapidly incorporated into liver and kidney. Kidney removed the greatest amount (21 μ mol/g wet wt.), about 10-fold more than heart, lung or liver. Lung and heart accounted for only 3%. These tissues then lost radioactivity during the remainder of the experiment. Radioactivity in the skeletal muscle, in contrast, increased from 8% of the injected dose at 5 min to 40% at 60 min. 4. In liver, kidney, heart and lung there was a significant difference in the fate of inosine. After initial incorporation of inosine, kidney predominantly lost inosine; heart preferentially lost purines; lung preferentially lost ribose radioactivity; and in liver the ribose radioactivity was rapidly lost, whereas purine was retained. Some of the ribose moiety was metabolized to glucose, presumably in the liver, and then released into the blood. Ribose radioactivity (probably as glucose) and radioactive hypoxanthine accumulated in skeletal muscle throughout the experiment. 5. Inosine caused a rapid and prolonged increase in the blood glucose content, from 6 to 15 mM in 60 min. This was accompanied by a small increase in plasma insulin. 6. It is concluded that the purine and ribose radioactivity lost from the kidney, liver and other tissues becomes incorporated into skeletal muscle.

Many tissues are able to remove purine compounds from the circulation and to incorporate them into their intracellular purine pools. The capacity of this mechanism is remarkably high, and compounds such as adenosine can elevate the intracellular ATP concentration of hepatocytes well above the normal value (Lund *et al.*, 1975). Organs such as heart, kidney and skeletal muscle release inosine or other purine compounds into the circulation during anoxia or other situations in which the phosphorylation state of their adenine nucleotides is decreased and nucleotide degradation is favoured (Alertsen *et al.*, 1958; Rubio *et al.*, 1974; Buhl, 1976).

Whatever its physiological role, the inosine-uptake mechanism has proved therapeutically useful. When inosine is administered to kidneys, it enables them to withstand ischaemia during surgery or transplantation (Fernando *et al.*, 1976, 1977*a,b*, 1979; Wickham *et al.*, 1978). Preliminary biochemical studies suggested that this was due to the provision of a pool of ATP precursors from which

the kidney could synthesize ATP when blood flow was resumed (Fernando *et al.*, 1976).

The purpose of the present study was to monitor the uptake of purine and ribose moieties of inosine into kidney and other organs after intravenous administration of double-labelled inosine and to measure the compounds into which they were incorporated. We found considerable exchange of inosine radioactivity among the organs within the time scale of the experiment. The liver, contrary to expectations (Murray, 1971), did not release significant amounts of radioactive purines into the circulation.

Materials and methods

[8-¹⁴C]Inosine and [2,5',8-³H]adenosine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [2,5',8-³H]Inosine was prepared from 20 nmol of [2,5',8-³H]adenosine (50 Ci/mmol) by incubating it with 0.1 mg of adenosine deaminase [EC 3.5.4.4; Boehringer Corp. (London), Lewes, Sussex, U.K.] in 1 ml of 0.1 M-triethanolamine buffer, pH 7.4, at 25°C for 50 min (Möllering & Bergmeyer, 1974). The purity of the

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product was checked by one-dimensional t.l.c. on silica gel in a butan-1-ol/ethyl acetate/methanol/conc. NH_3 (sp.gr. 0.880) (7:4:3:4, by vol.) solvent system (Pull & McIlwain, 1972; Sharma, 1981). Less than 1.5% radioactive adenosine remained. Inosine was obtained from Sigma Chemical Co., Poole, Dorset, U.K.; AnalaR grades were used when obtainable.

Male Porton Wistar rats (approx. 200 g) were purchased from Bantin and Kingman Ltd., Hull, U.K., and fed on Oxoid 41B diet in our animal house 5–10 days before the experiment. They were anaesthetized with sodium pentobarbitone (60 mg/kg, intraperitoneally) (May and Baker, Dagenham, Essex, U.K.), and 1 ml of a solution containing [$8\text{-}^{14}\text{C}$]inosine (3 μCi) and [$2,5',8\text{-}^3\text{H}$]inosine (17 μCi) together with 40 mg of unlabelled inosine in 0.9% NaCl solution was injected into the inferior vena cava.

Three batches of rats were used. In the first, the samples were taken in sequence at each of the stated times. A blood sample was taken and deproteinized in 1 vol. of 10% (w/v) trichloroacetic acid. The other tissues were rapidly freeze-clamped with liquid- N_2 -pre-cooled tongs in the following sequence: leg muscle, kidney, liver, heart and lung, within 90 s. The frozen tissues were powdered and homogenized in 5 vol. of 10% trichloroacetic acid in 20% (v/v) methanol solution. Radioactivity in the acid extracts was measured. A correction was made for the blood content of each tissue, measured under identical conditions (Sharma & Rodrigues, 1981). The ^{14}C -labelled purines and their degradation products in each tissue were separated by t.l.c. on silica gel (Pull & McIlwain, 1972; Sharma, 1981) and their radioactivity was measured. The amount of purine was too low to be measured chemically by high-performance liquid chromatography, so specific radioactivities could not be determined.

The amount of ribose present in each sample was determined from the ^3H radioactivity, but it was necessary to make a correction for the loss of ^3H from the C-2 during conversion of hypoxanthine into xanthine and from C-8 during conversion of xanthine into uric acid. The measured concentrations of these compounds were used to correct the ^3H radioactivity to give the radioactivity corresponding to ribose products. Although this is referred to as ribose, it should be noted that ribose was not chemically identified. Skeletal muscle was assumed to be 40% of body weight (Munro, 1969).

In the second experiment, sequential 0.7 ml blood samples were taken into heparinized tubes at 5, 10, 20, 40 and 60 min. After the removal of the 20 min samples, 1 ml of 0.9% NaCl was injected to maintain the animal's blood volume. A sample of the blood was deproteinized with 9 vol. of 3% (w/v) HClO_4 and the glucose content was determined enzymic-

ally (Elliott *et al.*, 1971). The erythrocytes and plasma were separated by centrifugation (2 min at 9500 g) and deproteinized with 2.5 and 1 vol. respectively of 10% trichloroacetic acid in 20% methanol. Radioactivity in the acid extracts was measured. Glucose in the neutralized plasma extract was separated by t.l.c. (Varley, 1967).

In the third experiment, the same amount of unlabelled inosine (160 μmol in 1 ml) was injected and blood samples were taken into heparinized tubes immediately before the injection and at 5, 15, 30 and 50 min. Control animals were injected with 1 ml of 0.9% NaCl. Glucose was determined in a portion of the blood, and the remainder was used to measure plasma insulin concentration by radioimmunoassay essentially as described by Hales & Randle (1963). Insulin was iodinated using the Iodogen method (Fraker & Speck, 1978). The insulin-binding reagent and the human insulin standard were obtained from Wellcome Laboratories (London SE13 6TL, U.K.).

^3H and ^{14}C in the acid extracts were determined simultaneously in an LKB Rackbeta scintillation spectrometer (LKB-Wallac, Bromma, Sweden), in a butyl-PBD [5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole; Koch-Light Laboratories, Colnbrook, Bucks., U.K.]/toluene (8 g of butyl-PBD/1 litre of toluene) scintillation fluid. Aqueous extracts (up to 0.1 ml) were solubilized with methoxyethanol (300 ml/litre of scintillation fluid). Efficiency of counting was assessed by a channels-ratio method (Baillie, 1960).

Results

Approx. 5% of the 160 μmol of inosine was incorporated within 2 min into the erythrocytes (Fig. 1a), and this radioactivity was retained for the duration of the experiment. Inosine removal by the other organs from the plasma was also rapid. More than 95% was cleared within 5 min, and 99% in 20 min (Fig. 1b). Nearly 70% of the inosine lost from the blood was taken up by the organs and tissues sampled; lung and heart only accounted for 3%, whereas liver and kidney over the first 10 min removed nearly 50% of the inosine lost from the blood.

The kidney initially took up the greatest amount of inosine, 21 $\mu\text{mol/g}$ wet wt., about 10-fold more than heart, lung or liver. Approx. 60% of this radioactive material was present as inosine, and the remaining 40% had been further metabolized (Fig. 2a). The intrarenal concentration of radioactive inosine decreased by 70% during the next 50 min, with no further increase in free ribose, xanthine or hypoxanthine, suggesting that it was lost as inosine. The small increase in uric acid can be explained by the decrease in xanthine and hypoxanthine concentrations. There was no significant loss of inosine

Fig. 1. Inosine metabolism in the erythrocytes and plasma

Doubly labelled inosine ($160 \mu\text{mol}$) was injected into the vena cava of anaesthetized rats. Erythrocytes and plasma were separated and deproteinized with HClO_4 (6%, w/v). ^{14}C and ^3H radioactivity was determined in the acid-soluble fraction. Purine base (∇) and ribose ($\blacktriangledown$) equivalents present were calculated for erythrocytes (a). By using the differential positions of the ^{14}C and ^3H in the inosine molecule, concentrations of inosine ($\blacksquare$), hypoxanthine ($\bullet$), xanthine ($\circ$), uric acid ($\triangle$) and free ribose ($\square$) equivalents were calculated for the plasma (b). The results are means $\pm$ S.E.M. for three to five observations.

smaller increase in uric acid concentration. There was also a net loss ($>70\%$) of the purine moiety of inosine and its degradation products from the heart, whereas the ribose moiety was retained.

In the lung (Fig. 3a), in contrast, from the earliest time point there was more than twice as much radioactivity from ribose in the tissue as from the purine moiety. The intracellular concentration of the latter was relatively constant at approx. 1.2 mM , whereas free ribose decreased during the remaining 50 min of the experiment from approx. 3 mM to approx. 2 mM .

Liver removed almost equal amounts of ribose and purine radioactivity in the first 5 min (Fig. 3b), indicating uptake as inosine. Little inosine was found in the tissue, however, suggesting that it had been rapidly metabolized. The ribose radioactivity was then rapidly lost, whereas purine radioactivity was retained. Because of its greater mass, the liver removed approximately the same proportion of the injected dose as the kidney, although the latter organ had a higher concentration.

In the skeletal muscle there was little initial uptake of inosine (Fig. 4), but subsequently large increases in the ribose and purine radioactivity occurred. Hypoxanthine was the main purine found.

An increase in blood glucose concentration after inosine injection was also observed (Fig. 5a). The increase was statistically significant from 5 min onwards. The control animals, which received an injection of 0.9% NaCl, showed no change in blood glucose concentration. The ^3H sugar in the blood was found, by t.l.c., to be glucose and not ribose. An increase in plasma insulin was also detected (Fig. 5b); it was statistically significant at 15 min after inosine injection.

Discussion

The organs studied showed marked differences in their uptake and metabolism of inosine. There was also evidence of exchange of inosine or its metabolic products between one tissue and another. The most probable degradative pathway for the labelled inosine would be conversion into $[5'\text{-}^3\text{H}]\text{ribose 1-phosphate}$ and $[8\text{-}^{14}\text{C}, 2,8\text{-}^3\text{H}]\text{hypoxanthine}$ catalysed by nucleoside phosphorylase (EC 2.4.2.1). After conversion of the ribose moiety into ribose 5-phosphate by an epimerase, the ribose ring could be oxidized via the pentose phosphate and glycolytic pathways and tricarboxylic acid cycle or the pentose phosphate pathway alone. Alternatively after metabolism in the pentose phosphate pathway to glucose 6-phosphate it could be converted into glycogen, lactate, pyruvate, alanine or glucose; the ^3H is retained in these molecules, despite the various interconversions involved. The last four substances are known to be exchanged between organs. Lac-

or its degradation products into the urine, since less than 0.5% of the radioactivity in the kidney at 10 min was found in the urine at 30 min. Labelled allantoin was not found in blood or any of the tissues examined.

Inosine uptake by the heart resembled that in the kidney, since at 2 min more than 50% of the radioactivity was present as inosine (Fig. 2b). Over the next 15 min, however, all of it had been metabolized intracellularly, with an equivalent increase in the concentration of free ribose and a

Fig. 2. *Inosine metabolism by kidney and heart in vivo*

Doubly labelled inosine (160 μmol) was injected into the vena cava of anaesthetized rats, and the radioactivity (^{14}C and ^3H) in the acid extract of tissue samples was determined. By using the differential positions of the ^{14}C and ^3H in the inosine molecule, concentrations of inosine (■), hypoxanthine (●), xanthine (○), purine nucleotide (▲), uric acid (△) and free ribose (□) equivalents were calculated per g of tissues. Results are means $\pm$ s.e.m. for three or four determinations.

Fig. 3. *Inosine metabolism by lung and liver in vivo*

For details and symbols see the legend to Fig. 2.

tate, pyruvate and alanine pass mainly from the skeletal muscle to other tissues; no loss of radioactivity from muscle was observed in these experiments, probably because the animals were relaxed under anaesthesia. Glucose is formed mainly by the liver. The marked rise in blood glucose after inosine injection (Fig. 5a) and the presence of [^3H]glucose in the blood suggest that ribose radioactivity lost from

the liver was released as glucose. This hypothesis has been supported by experiments (R. J. Sharma & J. R. Griffiths, unpublished work) in which livers perfused with 10mM-inosine raised the concentrations of glucose in the perfusion medium from 5.5 ± 0.7 to 15.5 ± 0.2 mM in 45 min.

The purine product of inosine phosphorolysis is hypoxanthine; this can combine with 5-phospho-

Fig. 4. Inosine metabolism by skeletal muscle *in vivo*. For details and symbols seen the legend to Fig. 2.

α -D-ribosyl diphosphate ('PRPP') to give IMP. IMP can be converted into AMP, ADP and ATP, or into the analogous guanine nucleotides, thus fixing the radioactivity within the intracellular purine pool. However, in the present experiments very small amounts were incorporated into the purine nucleotides in any organ. Purine radioactivity can be lost from the tissues as inosine, hypoxanthine or their degradation products, xanthine, uric acid and allantoin. Inosine, xanthine and hypoxanthine can be taken up by the other tissues, whereas uric acid and allantoin are excreted in the urine. There was no evidence of significant loss of uric acid or allantoin (or of any other purine) in the urine during the experiment, and the concentrations of hypoxanthine and xanthine fell slightly from their values at 10 min.

It is clear from the present data that both ribose and purine radioactivity is exchanged among organs and tissues during the experiment. The rise in radioactivity in skeletal muscle between 10 and 60 min is much greater than the loss of radioactivity from the blood in this period; uptake of inosine metabolites released by other organs, principally liver (as glucose) and kidney, accounts for the difference. Heart and lung also lost radioactivity during this period, but the principal loss from heart was purine, whereas lung lost ribose radioactivity almost exclusively. The nature of purine material being taken up by skeletal muscle is not clear, since an increase in the plasma concentration of the likely candidates such as inosine, hypoxanthine or xanthine was not observed. However, only [^{14}C]hypoxanthine was isolated in appreciable amounts from skeletal muscle. Kidney was unique in that it appeared to lose inosine, whereas other tissues lost inosine metabolites. However, no increase in plasma

Fig. 5. Effect of inosine on concentrations of blood glucose and plasma insulin *in vivo*.

The inferior vena cava of Nembutal-anaesthetized rats was exposed and a blood sample (0.6 ml) was removed into a heparinized tube. Inosine solution (1 ml; 160 mM) was injected into the vena cava and subsequent blood samples were removed at the intervals indicated. Control animals were injected with 0.9% NaCl. Glucose (a) and insulin (b) were measured as described in the Materials and methods section. Filled symbols ($\blacktriangle$, $\bullet$) represent glucose and insulin concentrations in inosine-injected animals and the open symbols ($\triangle$, $\circ$) concentrations in control animals. Results are expressed as means $\pm$ S.E.M. for three or four observations. A unit is 40 μg of insulin. * $P < 0.05$ by Student's t test.

inosine concentration was observed, indicating that it was either metabolized as it was transported out of the kidney or immediately after its release or that it was very avidly taken up by other tissues.

The rise in plasma insulin concentration (Fig. 5b) could have been caused by a direct effect of inosine on the cells of the islets of Langerhans (Campbell & Taylor, 1977) or by the rise in blood glucose (Fig. 5a).

It has been generally supposed, mainly on indirect evidence, that the liver is a main site of purine synthesis within the body and that purines carried from it by the blood are taken up by other tissues (see review by Murray, 1971). The present data do not support this hypothesis. No significant loss of purine radioactivity was observed from the liver in the 1 h period of the experiment, and the only other organs that lost purines were heart and kidney. The only tissue that gained purine in the latter part of the experiment was skeletal muscle. This appeared to act as a 'sink' for both the ribose and purine radioactivity.

Two points, however, should be noted. The first is that a large dose of unlabelled inosine was given along with the radioactive tracer, since the primary aim of the experiment was to assess the metabolism of pharmacological doses of this nucleoside. It is possible that the purine pool formed in the liver and other tissues under these conditions behaves differently from the physiological pool. Secondly, the duration of the experiment was limited because the rats were kept under anaesthesia. A longer-term experiment would have detected purine loss from liver (see Henderson & LePage, 1959; Smith & Salmon, 1969; Pritchard *et al.*, 1970, 1975).

Although no incorporation of inosine radioactivity into kidney nucleotides was observed in the present experiments, there is evidence (Fernando *et al.*, 1976) that more rapid ATP production takes place during recovery from ischaemia in kidneys pre-treated with inosine. Since kidney takes up more inosine per gram of tissue than any other organ, it is clear that intravenous administration of inosine to supplement the purine pool is particularly suited to the treatment of kidneys that are to be made ischaemic. The rapid loss of radioactivity after 10 min, however, shows that the timing of the pretreatment is critical. Fortunately inosine was given intravenously at 10 or 20 min before induction of renal ischaemia in earlier studies on its efficacy as a preservative (Fernando *et al.*, 1977b). Most of the injected dose would thus have been present at the onset of ischaemia.

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