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A COMPARATIVE STUDY OF PURINE METABOLISM BY HUMAN AND PIG ERYTHROCYTES *IN VITRO*

BETTY M. DEAN

The Medical Professorial Unit, St. Bartholomew's Hospital, London, E.C.1.

H. ANNE SIMMONDS

Department of Medicine, Guy's Hospital, London, S.E.1, England

and

ANDREW CADENHEAD

The Rowett Research Institute, Bucksburn, Aberdeen, Scotland

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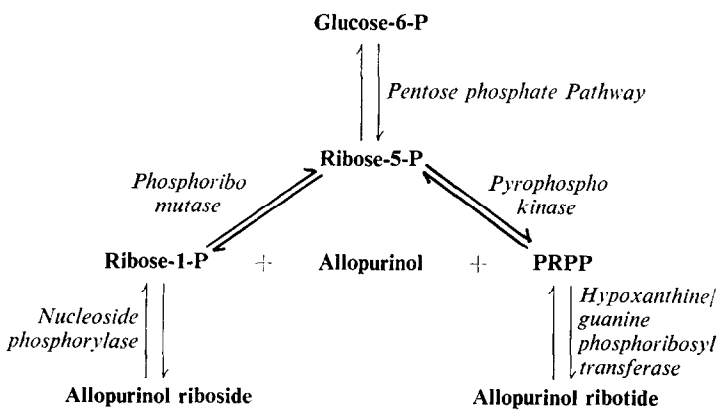
Abstract—Comparative rates of riboside and ribotide formation have been studied in pig and human intact and haemolysed erythrocytes *in vitro* by means of [^{14}C]labelled bases and electrophoretic separation of metabolites. In a physiological buffer system, ribosides were the major metabolites of hypoxanthine, guanine and xanthine in human intact cells, but pig erythrocytes showed little formation of metabolites in this system. When ribose-1-phosphate was added to a medium employing ribose in place of glucose, nucleoside formation by the pig erythrocytes exceeded that of the human cells, but the order of specificity, Gu > Hx > X > Allopurinol was common to both and no adenosine was formed by either. In either buffer system in both human and pig intact erythrocytes nucleotide formation was exceedingly low and allopurinol ribotide was not detectable. When intact cells were replaced by an equivalent volume of haemolysed cells, however nucleotides formed the major metabolites in both species in the presence of PRPP. In the pig erythrocyte PRPP and ribose-1-phosphate levels were exceedingly low and likewise the pig cell was capable of synthesizing these compounds at only minimal rates compared with humans. These results are discussed in relation to studies concerning the metabolic fate of allopurinol in pigs.

ALLOPURINOL (4-hydroxypyrazolo-(3,4-d)pyrimidine) is now widely used in man in the treatment of gout and hyperuricaemia. It has been shown to be a substrate for the



enzyme purine nucleoside phosphorylase (purine nucleoside: orthophosphate ribosyl transferase EC. 2.4.2.1) in both intact and haemolysed human erythrocytes,[1] In addition it is a known substrate for the enzyme IMP: pyrophosphate phosphoribosyl transferase (EC 2.4.2.8) in haemolysed cells.[2]

Despite the fact that allopurinol ribotide formation has been demonstrated only *in vitro*[3,4] its formation *in vivo* is tacitly assumed.[4–6] However, recent metabolic experiments in pigs have failed to reveal incorporation of allopurinol into tissue nucleotides *in vivo*.[7] Since studies of this type are not possible *in vivo* in man, we have investigated concurrently *in vitro* purine and pyrozalopyrimidine metabolism in pig erythrocytes. For extrapolation to man similar *in vitro* studies have been carried out in human erythrocytes.

In the intact erythrocytes two separate systems were used and, since the pig red cell shows a relative impermeability to glucose *in vitro*[8] these systems have been compared using both glucose and ribose, which is an effective substrate for ATP maintenance in the pig erythrocytes.

The first system (A) was used to study comparative riboside and ribotide formation at endogenous rates i.e. using physiological levels of purine bases in a buffer system analogous to human serum.

The second system (B) employed conditions designed to favour maximum nucleoside formation in order to assess comparative affinities of the purine bases for purine nucleoside phosphorylase.

In addition comparative rates of nucleotide formation have been studied in haemolysed erythrocytes, using conditions designed to favour maximum nucleotide synthesis.

EXPERIMENTAL

Materials. Hypoxanthine-8[^{14}C] guanine-8[^{14}C] sulphate, adenine-8[^{14}C] were obtained from The Radiochemical Centre, Amersham, England. 2[^{14}C]-xanthine from Schwarz Bioresearch, New York and 6-[^{14}C]-allopurinol (sp. act. 4·53 mCi/mmole) was kindly provided by courtesy of The Wellcome Foundation, England. Earle's BSS Medium (10 × concn W/O Phenol Red and Glucose) was obtained from Wellcome, England.

Sample collection. Blood was drawn at regular intervals from the superior vena cava of three pigs (A, B, C, litter mates) throughout the 8-week period of the metabolic study.[7] Pig C acted as the control, pig A was initially treated with allopurinol, pig B with guanine. Subsequently both pigs A and B were maintained on a mixture of both drugs. Pig D was an unrelated pig maintained on the same control diet as pig C.

The human subjects (1, 2, 4) were normal healthy adults on an unrestricted diet, whilst subject 3* was a gout patient, hospitalized for treatment of obesity, and whose hyperuricaemia had been successfully controlled by allopurinol therapy for the past 2 yr. In all cases blood was taken 2 hr after the morning meal; drug supplements in the pig studies and patient 3* being given with the meal.

Methods

Preparation of cells. Blood was drawn into heparinized tubes and the cells separated immediately from plasma and leucocytes by centrifugation. Cells were washed once with an equal volume of saline and twice with the buffer appropriate to the system

under study. Centrifugation time was extended and speed was reduced in the pig studies, because of the relative fragility of pig red cells.[9]

Incubation procedure. Freshly washed cells (1 ml) were pre-incubated for 15 min at 37° with the appropriate buffer system (3·5 ml) and the reactions started by addition of the ^{14}C base in buffer (0·5 ml) (the final haematocrit of the mixture was 20 per cent). 0·5 ml samples were removed at 0, 0·75, 1·5 and 2·25 hr and the reactions stopped by immediate centrifugation (5 min at 2500 rev/min) and the addition of 5% $HCLO_4$ (100 μl to the supernatant) and 2·5% $HCLO_4$ (500 μl to the cells). After thorough mixing, cell extract and medium were again centrifuged and the clear supernatants stored deep-frozen prior to electrophoresis.

System A. A buffer (pH 7·4) comparable to human serum was obtained by dissolving Earle's medium (10 ml), crystallized bovine plasma albumin (1 g), $NaHCO_3$ (168 mg), and D-glucose (100 mg) in water (100 ml). This buffer was used in the incubation procedure without the addition of ribose-1-P and reactions were started by the addition of the requisite ^{14}C base to give a final concentration of 0·05 mM.

System B. The buffer for this system was essentially the same as that employed in System A except that the D-glucose was replaced by D-ribose (83·4 mg). In this system the cells (1 ml) were pre-incubated with this buffer (3 ml) and α-D-ribose-1-phosphate dicyclohexylammonium salt (5 mM, 0·5 ml in buffer) and the reactions started by adding the appropriate ^{14}C base in 0·5 ml buffer to give a final concentration of 0·1 mM base.

Haemolysed cell experiments. Cells were separated and washed in the same manner as for the intact cell experiments. Haemolysis was affected by rapid freezing (at approximately −70°) and thawing three times, followed by centrifugation to remove cell ghosts. Comparative experiments for intact and haemolysed erythrocytes employed equivalent volumes of the two cell preparations in buffer system A using ^{14}C base and PRPP at a final concentration of 0·1 mM and 1 mM, respectively.

Estimation of metabolites. The bases, nucleosides and nucleotides were separated by co-electrophoresing aliquots of the medium and cell extracts with 0·1 μmole of appropriate cold carrier markers (80 V/cm, Whatman 3 mM paper in borate buffer (0·05 M pH 9·6) containing EDTA (0·01 M) for 30 min). The metabolites were located using an u.v. light source (254 nm), cut out, and immersed in 15 ml of toluene containing scintillator butyl-PBD (0·5% w/v). Radioactivity was measured in a liquid scintillation spectrometer (Nuclear Chicago, Mark I at 60–65 per cent efficiency).

Phosphoribosyl pyrophosphate (PRPP) and ribose-1-P estimations. The method used was essentially that of Sperling *et al.*,[10] which relies upon added 2,3-diphosphoglyceric acid to inhibit further PRPP synthesis throughout the course of the estimation.

We employed 8[^{14}C]-hypoxanthine and the PRPP assay buffer described by these authors, but estimated the inosine and IMP formed by the electrophoretic method and scintillation counting system described above.

The ability of the pig and human red cells to synthesize ribose-1-P and PRPP was measured by repeating the estimation of inosine and IMP formation in cells that were pre-incubated for 2 hr in Earle's medium as made up for System A, or System B and containing added sodium phosphate (12 mM).

RESULTS AND DISCUSSION

The pig red cell differs from that of the human in its apparent inability to glycolyse *in vitro* because the cells have a very low permeability to glucose. Ribose and deoxy ribose have been shown to be effective substrates for the maintenance of ATP in the intact pig erythrocyte[8] and it is known that ribose-5-phosphate, inosine and adenosine can produce lactate in the pig red cell.[11–13] Because of this difference between the human and pig red cell we have studied comparative nucleoside and nucleotide formation in two systems. Firstly, System A at a physiological level of purines in a buffer system analogous to human serum, and secondly, System B using conditions designed to favour maximum nucleoside formation, in order to assess inhibition of purine nucleoside phosphorylase by allopurinol.

TABLE 1. COMPARATIVE RIBOSIDE AND RIBOTIDE FORMATION BY INTACT PIG AND HUMAN RED CELLS USING SYSTEM A (METHODS SECTION)

Substrate		Riboside formation (nmole/hr/ml red cells)				Ribotide formation (nmole/hr/ml red cells)			
		Hx	Gu	Ad	X	Hx	Gu	Ad	X
Pig	A	0·42	0·32	0·30	0·04	1·10	0·36	1·12	0
	B	0·68	0·38	0·20	0·08	0·66	0·30	0·66	0
	C	0·38	0·50	0·22	0·06	0·84	0·46	0·72	0
Human	1	17·6	20	0	1·6	0·66	1·0	1·5	0
	2	18·6	21	0	1·0	1·76	1·28	0·44	0
	3*	17·2	20	0	1·2	1·90	3·40	1·36	0

Pig C was the control pig and these measurements on pigs A and B were made prior to any drug treatment. Human subjects (1, 2, 3*; subject 3* on allopurinol) are those previously described (see Experimental section).

At physiological substrate levels applicable to the human red cell, ribosides were the major metabolites of hypoxanthine, guanine and xanthine in the three humans studied (Table 1), the corresponding formation of ribotide being exceedingly low and, in fact not measurable for xanthine. In the pig intact erythrocyte, however, although the rates of ribotide formation were likewise exceedingly low and comparable to those of the human (and XMP was again not measurable), inosine and guanosine were formed at only a fraction of the human rate. With adenine as substrate, (ADP + ATP) were the major metabolites for pig and human cells, very little adenosine being formed by the pig cells and none at all in the human. Values for nucleotide formation shown in Table 1 represent total mono-, di- and tri-nucleotides, and no attempt was made to evaluate any interconversion. In both the pig and human studies (ADP + ATP) formation was consistently in excess of AMP formation, whereas with hypoxanthine or guanine as substrate, IMP and GMP formed the major nucleotide component in this system.

The formation rates shown in Table 1 refer to values found in the cell extract only. It is of interest here that administration of allopurinol to subject 3* did not appear to alter the rates of either riboside or ribotide formation as compared to the normal subjects 1 and 2. Under these experimental conditions no transport effects were observed for any of the purines studies, in that the percentage of riboside formed in the cell extract and the corresponding medium was always essentially identical, and

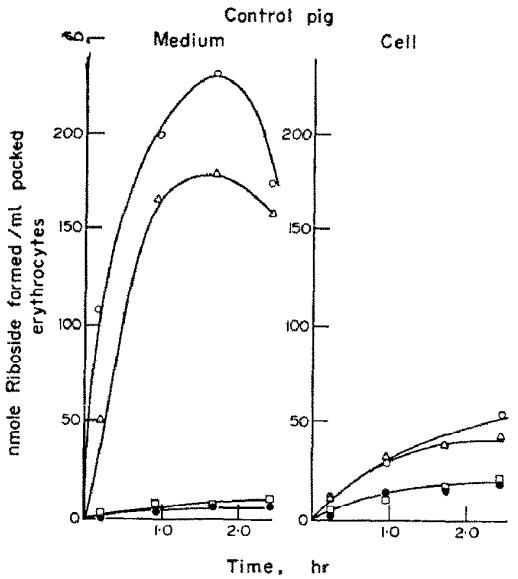


FIG. 1

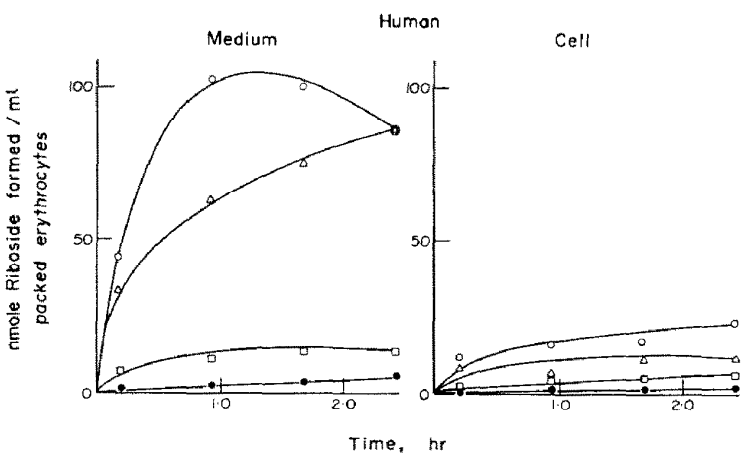


FIG. 2

FIGS. 1 and 2. Comparative rates of riboside formation in the medium and cell extract using System B, (Methods section) in erythrocytes from a control pig and a normal human (subject 1) respectively (haematocrit 20 per cent) ○; △; □; and ● show rate of formation of guanosine, inosine, xanthosine and allopurinol riboside respectively from 500 nmoles of the corresponding base.

the radioactivity levels of the medium and cell extract were equivalent. In no instance was there any leakage of nucleotide from the cell into the medium, but since the experiments were performed at a haematocrit value of 20 per cent, values for the total conversion of base to corresponding nucleosides (i.e., cell extract + medium values) will be approximately five times those shown in Table 1.

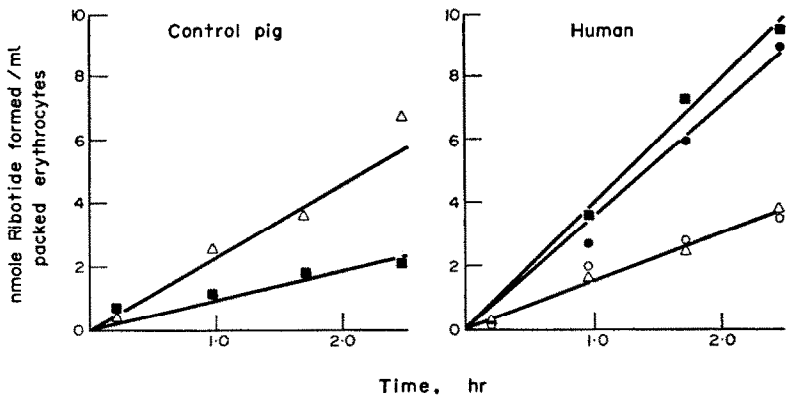


FIG. 3. Comparative rates of ribotide formation in the control pig and normal human intact erythrocyte, ○; ●; ▲ and ■ show formation of GMP (GDP + GTP), IMP and (ATP + ADP) respectively formed in the cell simultaneously with the corresponding nucleosides shown in Figs. 1 and 2.

From these results it would appear that the enzyme purine nucleoside phosphorylase plays the more significant role in controlling the metabolic fate of the purines hypoxanthine and guanine in the intact erythrocyte, whereas the enzymes Hx/Gu PRTase and Ad PRTase appear to exert a comparatively minor influence under these simulated physiological conditions.

Figures 1 and 2 demonstrate clearly, however, that in the presence of adequate ribose-1-phosphate in the medium (System B), the pig erythrocyte shows a faster conversion rate to the nucleoside for all the purines studied than the human. No adenosine was formed in either the human or pig erythrocyte under these conditions, indicating that the low levels of adenosine formed by the pig in Table 1 are due to the breakdown of adenine nucleotides by 5′-nucleotidase, (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5). It is of interest that at equimolar substrate levels, allopurinol is the poorest substrate for nucleoside phosphorylase in both human and pig cells, its specificity being similar to that of xanthine.

In this system in the human, IMP and GMP were formed at a comparable rate to IMP in the pig (Fig. 3), but we were unable to demonstrate GMP formation in repeated studies on the pigs (A), (B) and (C). Measurements of GDP, GTP, IDP, ITP were not made in these pig studies, but in the three human studies (IDP + ITP) was not significant, whilst (GDP + GTP) was formed at approximately × 2 the rate of GMP. The rate of (ADP + ATP) formation in the human was approximately × 2 that found in the pig, whilst no AMP, XMP or allopurinol ribotide formation could be detected in either the human or pig extracts using System B.

Figure 4 shows that in System B, as in System A, all the bases studied readily entered the cell. Since experiments were performed at a haematocrit value of 20 per cent, with 500 nmole base in the medium initially, if the bases were transported freely into the cell without metabolism, the expected level would be 100 nmole in the cell and 400 nmole in the medium. Xanthine remained in the medium at the highest level and appeared in the cell at the lowest level in both human and pig studies, whereas levels of allopurinol in both cell and medium were comparable to those for adenine and higher than hypoxanthine. The only major difference betweeen pig and human

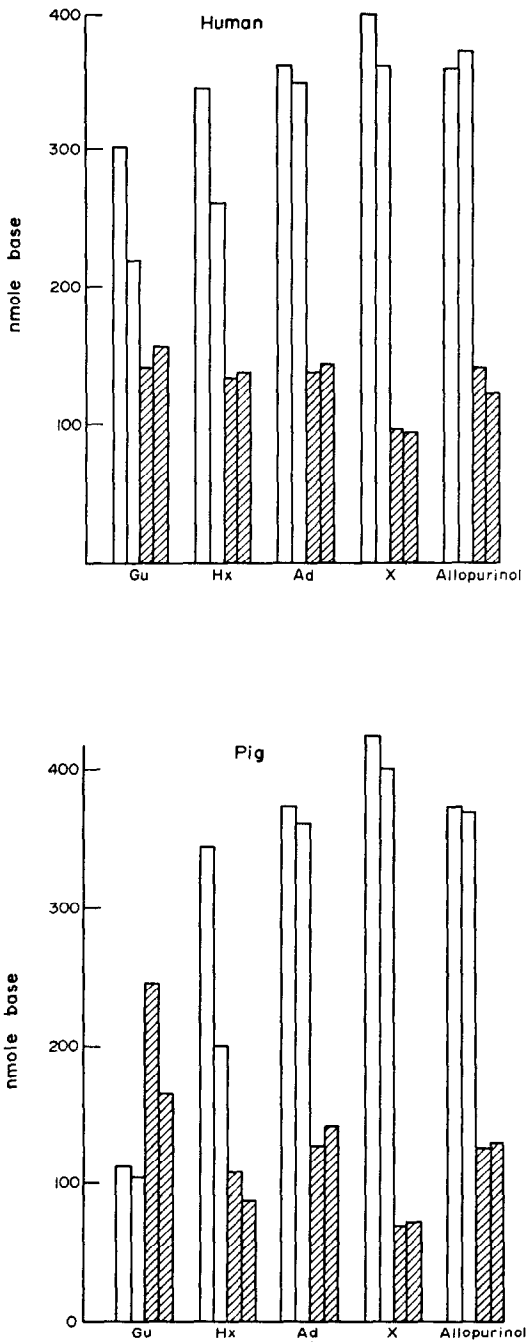


FIG. 4. Distribution of Gu, Hx, Ad, X and allopurinol between the medium (□) and cell (▨) for normal human (1) and control pig (C) using system B (Methods section). Consecutive levels for each base correspond to the initial and final time points as shown for the relevant nucleoside (Figs. 1 and 2) and nucleotide formation (Fig. 3).

results presented in Fig. 4 is for guanine, which appeared in the pig erythrocyte at approximately twice the level in the medium, a surprising finding since GMP was not measurable here. Because of the low levels of guanine in the media, electrophoretograms were examined for other metabolites, particularly hypoxanthine and inosine, since 8[^{14}C]hypoxanthine was detected in the urine of these pigs when fed 8[^{14}C]guanine. However, in agreement with Audy *et al.*,[14] no evidence of guanase (EC 3.5.4.3) activity was found and no other major metabolites were detected.

Serial studies throughout the 8-week period of the metabolic experiment[7] showed that during combined feeding with allopurinol and guanine, erythrocytes from pigs (A) and (B) had a reduced capacity for nucleoside formation (< 20 per cent that of control pig C) using hypoxanthine, guanine, xanthine or allopurinol as substrates. Simultaneous formation of IMP, however, was not reduced. At this time the pigs were in renal failure following an acute crystal nephropathy, and urinary allopurinol riboside excretion was considerably reduced. The capacity for nucleoside formation returned to control levels when the studies were repeated four weeks after drug treatment had ceased. The observed inhibition was not due to impaired transport of the bases into the cell as the cell–medium distribution of the individual bases was unaltered and similar to that of the control pig (Fig. 4).

Throughout these studies we have failed to demonstrate the formation of allopurinol ribotide in any of the systems employing intact erythrocytes. This is in agreement with Iwata *et al.*[15] who have recently demonstrated *in vitro* that allopurinol is not a competitive inhibitor of Hx/Gu PRTase.

Human and pig intact erythrocytes in a physiological buffer (System A) with added PRPP (1 mM) showed no conversion of allopurinol to either the riboside or the ribotide. (cf. the formation of allopurinol riboside shown in Figs. 1 and 2, using 1 mM ribose-1-phosphate in the medium. Although this difference may be attributable to the relative inabilities of PRPP and ribose-1-phosphate to cross the cell membrane, formation of IMP under these conditions (although low compared with inosine formation in the presence of 1 mM ribose-1-phosphate), was considerably greater than when PRPP was omitted from the assay (see Table 1). When haemolysed cells were incubated under the same conditions with 1 mM PRPP, allopurinol ribotide was the main metabolite of allopurinol and hypoxanthine was almost totally converted to IMP by both human and pig haemolysates (Table 2).

TABLE 2. COMPARATIVE RIBOSIDE AND RIBOTIDE FORMATION (nmole/hr/ml RED CELL) BY INTACT (I) AND HAEMOLYSED (H) HUMAN AND PIG ERYTHROCYTES USING BUFFER SYSTEM A, 0·1 mM BASE and 1 mM PRPP

		Inosine	IMP	Allopurinol riboside	Allopurinol ribotide
Human (1)	(I)	105	23	0	0
	(H)	1	495	30	45
Pig (C)	(I)	30	35	0	0
	(H)	0·5	495	15	155

The results of the experiments designed to measure erythrocyte levels of PRPP and ribose-1-phosphate indicate that the pig cells have considerably lower endogenous

levels of these compounds (1/10 human), and are able to synthesize them at only a fraction of the human rate (Table 3). Although this can be attributed to the inability of the intact pig cell to glycolyse *in vitro*,[8] the rate of synthesis of either compound was not greatly elevated in the pig cell by substituting ribose for glucose in the medium.

TABLE 3. COMPARISON OF PIG AND HUMAN ERYTHROCYTE PRPP AND RIBOSE-1-PHOSPHATE LEVELS AND OF THEIR ABILITY TO SYNTHESIZE THOSE COMPOUNDS IN THE PRESENCE OF 5 mM GLUCOSE OR 5 mM RIBOSE

		Erythrocyte levels (nmole/ml packed cells)		Erythrocyte synthesis (nmole PRPP/2 hr/ml packed cells)		(nmole ribose-1-P 2 hr/ml packed cells)	
		PRPP	Ribose-1-P	Glucose medium	Ribose medium	Glucose medium	Ribose medium
Pig	A	0·1	0·3				
	C	0·4	0·4	0·5	0·7	0·5	0·7
	D	0·2	0·8	1·3	1·4	1·1	1·9
Human	1	1·4	5·4	51·8	58·6	3·3	4·2
	2	1·4	3·5	40·4		2·6	
	4	2·3	4·5	46·2		4·5	

Pigs A, C, D, and humans 1, 2, 4, are those previously described in the experimental section (see Sample collection).

Pig blood samples were also analysed for PRPP and ribose-1-phosphate at intervals throughout the course of the metabolic experiment[7] but because of the exceedingly low levels, the assay was not sensitive enough to attach any significance to changes due to the metabolism of these drugs (cf. Fox *et al.*[4] who demonstrated a reduction in PRPP levels in the human erythrocyte following a single dose of allopurinol).

Despite low erythrocyte levels of PRPP and ribose-1-phosphate the pig has an apparently unlimited capacity to convert allopurinol to allopurinol riboside *in vivo*,[7] which indicates that it is the availability, rather than the actual erythrocyte levels of these compounds which is of greater significance *in vivo*. In view of this it is suggested that *in vitro* experiments on the red cell, particularly haemolysates, may afford misleading results with regard to the overall *in vivo* metabolism of purines and their analogues.

Although the pig red cell is unable to glycolyse *in vitro*, these studies demonstrate that it undoubtedly possesses enzymes concerned with purine metabolism similar to those found in the human erythrocyte. In both species it would appear that purine nucleoside phosphorylase plays a regulating role for the availability of the purines hypoxanthine and guanine for further metabolism.

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