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Transfer of 1-pyrroline-5-carboxylate as oxidizing potential from hepatocytes to erythrocytes

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The interconversions of proline and 1-pyrroline-5-carboxylate form an intercellular cycle that is the basis of a metabolic interaction between hepatocytes and erythrocytes. The cycle transfers oxidizing potential from hepatocytes to erythrocytes, which stimulates pentose phosphate pathway in erythrocytes. This interaction depends on the differential metabolism of proline and 1-pyrroline-5-carboxylate in erythrocytes and hepatocytes and consists of the following: in hepatocytes proline oxidase converts proline into 1-pyrroline-5-carboxylate, which is released into the medium and taken up by erythrocytes; erythrocyte 1-pyrroline-5-carboxylate reductase converts 1-pyrroline-5-carboxylate into proline and concomitantly generates $NADP^+$; the generated oxidizing potential drives glucose metabolism through the pentose phosphate pathway in erythrocytes; finally, erythrocytes release proline into the medium, enabling it to re-enter hepatocytes and repeat the cycle. The increased activity of the pentose phosphate pathway in erythrocytes may enhance the production of 5-phosphoribosyl pyrophosphate, a necessary moiety for the processing of purines.

Studies of proline metabolism in erythrocytes and hepatocytes have led us to hypothesize a metabolic interaction between these two cells that transfers oxidizing potential from hepatocytes to erythrocytes. The metabolic interaction is based on the differential metabolism of proline and P5C in hepatocytes and erythrocytes and its immediate consequence is a marked stimulation of pentose phosphate-pathway activity in erythrocytes.

The key enzymes involved in the metabolic interaction are proline oxidase (EC number not assigned) and P5C reductase (EC 1.5.1.2). Proline oxidase is tightly bound to mitochondrial inner membranes and supports oxidative phosphorylation (Johnson & Strecker, 1962; Kramar, 1967; Adams, 1970; Meyer, 1977; Scriver, 1978; Adams & Frank, 1980). As proline is oxidized to P5C, electrons are donated to mitochondrial electron transport presumably through a flavoprotein. P5C reductase, a cytosolic enzyme, converts P5C into proline with concomitant oxidation of NADPH or NADH (Scheme 1) (Smith & Greenberg, 1957; Peisach & Strecker, 1962; Yeh & Phang, 1980).

The differential distribution of proline-metabolizing enzymes in mammalian tissues underlies the proposed metabolic interaction. P5C reductase is widely distributed in mammalian tissues (Herzfeld *et al.*, 1977). Erythrocytes have a high P5C reductase activity, but proline oxidase, P5C dehydrogenase and ornithine aminotransferase are undetectable (Yeh & Phang, 1980). Thus within erythrocytes the only metabolic fate of P5C is its conversion into proline. In contrast hepatocytes contain not only P5C reductase and proline oxidase, but also ornithine aminotransferase and P5C dehydrogenase, which catalyse the conversions of ornithine into P5C and P5C into glutamate respectively (Adams, 1970). Consequently, hepatocytes can produce P5C and P5C can be converted into ornithine, glutamate or proline, depending on regulation of the respective enzymes (Scheme 1).

The differential response of hepatocytes and erythrocytes to P5C is another important factor underlying the metabolic interaction. P5C added to the incubation medium serves as a potent oxidizing equivalent for erythrocytes. Previous observations have indicated that P5C markedly stimulates glucose metabolism in the pentose phosphate pathway in erythrocytes (Yeh & Phang, 1980) as well as in cultured fibroblasts (Phang *et al.*, 1979). In contrast, neither proline nor P5C stimulates the hepatocyte pentose phosphate pathway (see below). This suggested to us that P5C, produced from proline in

Abbreviation used: P5C, 1-pyrroline-5-carboxylate.

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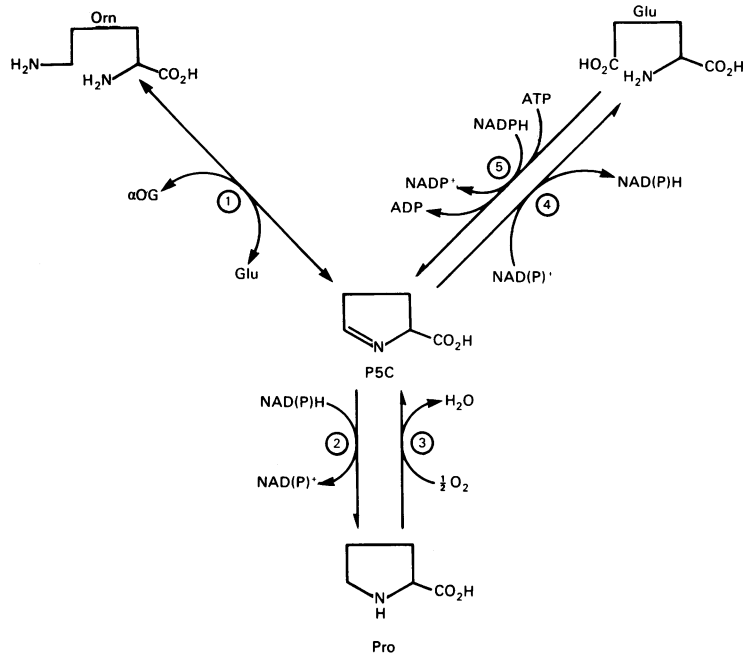


Scheme 1. *Pathways of P5C synthesis and degradation*
Enzymes are designated as: (1) ornithine aminotransferase; (2) P5C reductase; (3) proline oxidase; (4) P5C dehydrogenase; (5) P5C synthase. The tissue location of specific enzymes is described in the text. Abbreviation used: αOG, α-oxoglutarate.

hepatocytes, may be released from hepatocytes as an oxidizing equivalent for other cells, e.g., erythrocytes. We set out to examine this question by co-incubating erythrocytes with isolated hepatocytes and using the generation of CO_2 from the C-1 carbon atom of glucose by the pentose phosphate pathway as a marker of this interaction. A preliminary report described our initial observations (Phang *et al.*, 1981), but important areas remained unexplored. We now report studies that (1) indicate that the interaction occurs at very short incubation times, (2) quantify the metabolic products of proline in hepatocytes, (3) demonstrate the relationship of the metabolic interaction to erythrocyte number, hepatocyte number and glucose and proline concentrations, (4) provide further evidence that P5C is the mediator of the metabolic interaction and (5) indicate that the metabolic interaction occurs in the absence of rotenone.

Materials and methods

Reagents

L-1-Pyrroline-5-carboxylate was synthesized enzymically and purified by a previously described method (Smith *et al.*, 1977). D-[1-^{14}C]Glucose (sp. radioactivity 45–60 Ci/mol) and L-[U-^{14}C]proline (>250 Ci/mol) were purchased from Amersham Radiochemicals and New England Nuclear respectively. Hyamine hydroxide was from New England Nuclear. All other reagents were obtained from Sigma or Baker Chemical Co.

Preparation of erythrocytes

Erythrocytes were prepared from heparinized venous blood of non-fasting normal volunteers. Cells were maintained at 4°C during the preparative procedure and were used within 3 h of venipuncture. After initial centrifugation (10 min at 4°C and 500 *g*) and removal of plasma, erythrocytes were washed three times with 5 vol. of 0.85% NaCl with special care taken to remove the buffy coat with each wash.

Isolated hepatocyte preparations

Rat hepatocytes were prepared from male Sprague–Dawley rats (150–200 g; Zivic Miller) by a previously described method (Seglen, 1973) and murine hepatocytes were prepared from male C-57/B6 or PRO/Re mice (3–4 months old from Jackson Laboratory) by a similar method with minor modifications (Moldeus *et al.*, 1978). All animals were starved for 24–48 h before procedures. Murine livers were perfused *in situ* through the portal vein using a syringe and 27-gauge needle

attached to a three-way stopcock with extension tubing. Initially livers were perfused for 5 min with 8 ml of Hanks balanced salt solution without Ca^{2+}, with 0.5 mM-EGTA and 2% bovine serum albumin. This was followed by 20 ml of Hanks solution with 0.12% collagenase (130 units/mg; Worthington) and 4 mM-$CaCl_2$ infused over 5 min. Livers were then removed and hepatocytes isolated in the same manner as in the rat. Isolated rat or murine hepatocytes were 85–95% viable as determined by Trypan Blue exclusion. Hepatocytes were gassed with O_2/CO_2 (19:1), maintained at room temperature before incubation and utilized within 30 min of the isolation procedure. The standard incubation conditions (see below) did not significantly affect the integrity of isolated hepatocytes. After various durations of incubation with or without proline, neither the total cell number nor the fraction of cells excluding Trypan Blue (85–95%) showed changes indicating significant hepatocyte disruption.

Incubation procedures

Cells were incubated in 25 ml Erlenmeyer flasks containing 1 ml of Earle's balanced salt solution, pH 7.4, with 2.5 mM-glucose, 0.5 μg of rotenone/ml unless specified otherwise, 1 μCi of [1-^{14}C]glucose and P5C or proline as indicated. Standard co-incubations contained 50 μl of erythrocytes and 4–6 mg (wet weight) of hepatocytes, unless specified otherwise. Flasks were gassed with O_2/CO_2 (19:1) immediately before incubation at 37°C in a metabolic shaker. Other specific conditions are indicated in the legends to Figures and Tables.

Quantification of $^{14}CO_2$ from [1-^{14}C]glucose

Liberation of $^{14}CO_2$ from [1-^{14}C]glucose was used to quantify the flux through the oxidative arm of the pentose phosphate pathway (Yeh & Phang, 1980). Incubations were terminated by addition of 0.3 ml of 3 M-H_2SO_4 and 0.3 ml of hyamine was added to centre wells to capture $^{14}CO_2$ produced from [1-^{14}C]glucose as described previously (Rosenberg *et al.*, 1961).

Quantification of $^{14}CO_2$ production from [U-^{14}C]-proline

The same method used to measure $^{14}CO_2$ production from [1-^{14}C]glucose was used to measure $^{14}CO_2$ released from [U-^{14}C]proline.

Quantification of P5C and proline

Unlabelled P5C was assayed colorimetrically using *o*-aminobenzaldehyde (Johnson & Strecker, 1962) and [^{14}C]P5C was measured by ion-exchange chromatography as previously described (Phang *et al.*, 1975). [^{14}C]Proline was also quantified by ion-exchange chromatography (Phang *et al.*, 1973).

Results

Experimental conditions

In hepatocytes treated with rotenone to inhibit mitochondrial NADH oxidation, the generation of $^{14}CO_2$ from [6-^{14}C]glucose oxidation was negligible and amounted to less than 0.3% of total $^{14}CO_2$ production from [1-^{14}C]glucose. Thus, in rotenone-treated hepatocytes little $^{14}CO_2$ originated from glucose oxidation in the tricarboxylic acid cycle and the pentose phosphate pathway was the sole source of $^{14}CO_2$ from [1-^{14}C]glucose. It was for this purpose that rotenone (0.5 μg/ml of incubation) was routinely used. As expected, erythrocytes lacking mitochondria did not generate $^{14}CO_2$ from [6-^{14}C]glucose (results not shown). Thus the generation of $^{14}CO_2$ from [1-^{14}C]glucose in both erythrocytes and rotenone-treated hepatocytes can be used to measure the flux through the oxidative arm of the pentose phosphate pathway.

Proline metabolic capacity of hepatocytes and erythrocytes

The pentose phosphate pathway in erythrocytes is stimulated by P5C but not by proline (Phang *et al.*, 1981). When erythrocyte pentose phosphate pathway was quantified by $^{14}CO_2$ production from [1-^{14}C]glucose, we found that 0.3 mM-P5C produced a greater than 4-fold increase over basal pentose phosphate-pathway activity. Under these conditions erythrocytes incubated with 0.3 mM-P5C released proline into incubation medium at a rate of 1.44 ± 0.10 μmol/h per ml of erythrocytes (mean $\pm$ S.E.M.) (Yeh & Phang, 1980). Additional studies showed that P5C in incubation medium was almost completely removed by erythrocytes. When incubated with 30% (v/v) erythrocytes and 2.5 mM-glucose, [^{14}C]P5C (0.05 mM) was rapidly converted into proline such that only 20% of P5C remained after 30 min and less than 10% remained after 60 min of incubation. Furthermore, proline was the only product of P5C recovered in these incubations. On the other hand, 5 mM-proline has no effect on the erythrocyte pentose phosphate pathway because erythrocytes contain no proline oxidase and are unable to produce P5C from proline (Yeh & Phang, 1980).

Hepatocytes show no change in pentose phosphate-pathway activity when incubated with 0.3 mM-P5C or 5 mM-proline (Phang *et al.*, 1981). Nevertheless, they readily converted proline into P5C and released P5C into the medium. Incubated with 5 mM-proline, hepatocytes released P5C into the incubation medium and the amount released increased with time (Fig. 1). Utilizing the colorimetric method for quantifying P5C concentration, we found that hepatocytes released P5C into incubation medium at a rate of 26.5 ± 1.4 μmol/h per g

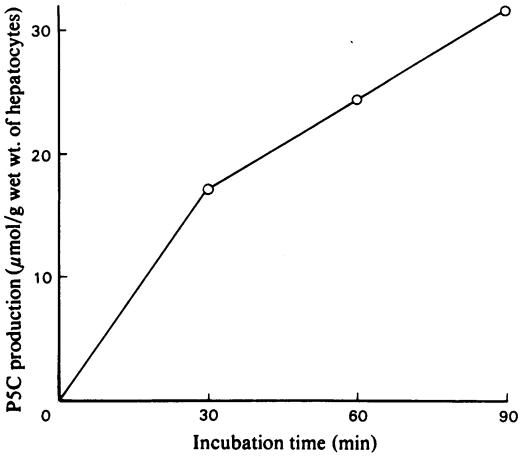


Fig. 1. *Production of P5C by rotenone-treated hepatocytes*
Hepatocytes (4–6 mg) were incubated in 1 ml of Earle's balanced salt solution with 2.5 mM-glucose, 0.5 μg of rotenone, and with 5 mM-proline. After incubation hepatocytes were removed by centrifugation (1 min in Beckman Microfuge B) and supernatants were assayed for P5C colorimetrically. Data represent averages of duplicate samples.

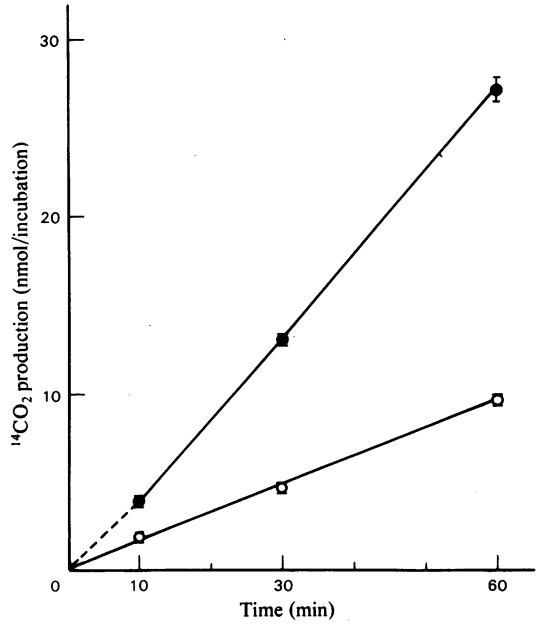


Fig. 2. *Effect of proline on $^{14}CO_2$ production from [1-^{14}C]glucose in hepatocyte/erythrocyte co-incubations*
Erythrocytes (150 μl) were co-incubated with hepatocytes (4–6 mg wet wt.) in 1 ml of Earle's balanced salt solution with 2.5 mM-glucose, 1 μCi of [1-^{14}C]glucose, 0.5 μg of rotenone and 5 mM-proline (●) or no proline (○). Data were normalized to 5 mg wet wt. of hepatocytes per incubation and are expressed as means ± S.E.M. of at least three determinations.

wet weight. Additional measurements of P5C release by hepatocytes were obtained by incubating hepatocytes with [U-^{14}C]proline (5 μCi) under the same conditions. [^{14}C]P5C was quantified by ion-exchange chromatography and found to be 27.0 μmol/h per g wet wt. of hepatocytes, thus verifying the measurements made with the *o*-aminobenzaldehyde method.

Stimulation of erythrocyte pentose phosphate pathway by co-incubating hepatocytes and erythrocytes with proline

We found that the addition of proline to co-incubations of hepatocytes and erythrocytes markedly stimulated erythrocyte pentose phosphate-pathway activity (Fig. 2). The addition of 5 mM-proline to co-incubations resulted in a significant increase in $^{14}CO_2$ liberation from [1-^{14}C]glucose by 10 min, and the amount of $^{14}CO_2$ liberated was linear between 10 min and 60 min. When hepatocytes were replaced with an equal volume of supernatant from final hepatocyte preparations, no proline-dependent increase in $^{14}CO_2$ was observed (results not shown). Since erythrocytes accounted for 97–98% of the total $^{14}CO_2$ produced in co-incubations without proline and P5C stimulated $^{14}CO_2$ production in erythrocytes but not in hepatocytes (Phang *et al.*, 1981), our working hypothesis was that hepatocytes converted proline into P5C, which then passed into erythrocytes, where its conversion into proline produced oxidizing potential for the pentose phosphate pathway.

Murine hepatocytes were chosen for these studies because of the availability of genetic mutant strains with abnormalities in proline metabolism. Nevertheless, the proline-dependent phenomenon occurred with rat hepatocytes as well as murine hepatocytes. The rat hepatocyte pentose phosphate pathway did not change with the addition of 5 mM-proline [2.81 nmol of $^{14}CO_2$/h per incubation without proline and 2.59 nmol of $^{14}CO_2$/h per incubation with proline (conditions the same as those described for Fig. 2, except erythrocytes were absent)]. However, 5 mM-proline added to incubations containing rat hepatocytes (5 mg) and erythrocytes (50 μl) produced a 2-fold increase in $^{14}CO_2$ release (6.41 ± 0.19 nmol of $^{14}CO_2$/h per incubation without proline and 13.63 ± 0.18 nmol of $^{14}CO_2$/h per incubation with proline).

We further characterized this metabolic interaction as a function of the quantity of hepatocytes or erythrocytes in co-incubations. Increasing volumes of erythrocytes from 10μl to 150μl in co-incubations containing 5mM-proline resulted in a linear increase in $^{14}CO_2$ production (results not shown). The addition of increasing quantities of hepatocytes to co-incubations containing proline resulted in a progressive increase in $^{14}CO_2$ production until a plateau was reached at 10mg of hepatocytes (results not shown). Although proline stimulated pentose phosphate-pathway activity at all glucose concentrations tested (0.1–5mM) in the standard co-incubation system, pentose phosphate-pathway activity remained a saturable function of glucose concentration and reached a plateau at 1mM-glucose with or without proline (results not shown). In co-incubations with constant hepatocyte weight and erythrocyte volume, the proline-dependent stimulation of the pentose phosphate pathway was a saturable function of proline concentration and reached a plateau at proline concentrations between 10 and 20mM (Fig. 3).

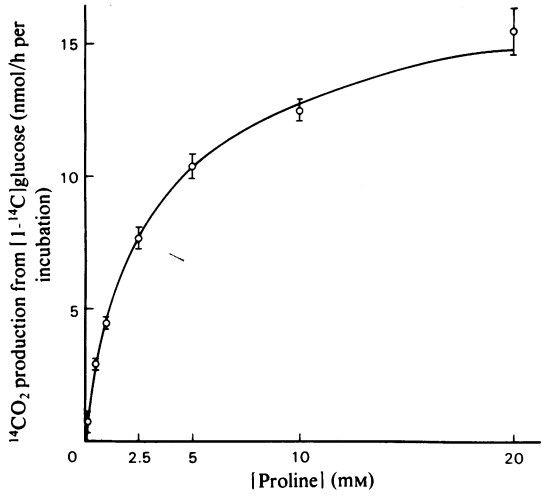


Fig. 3. *Effect of increasing proline concentration in hepatocyte/erythrocyte co-incubations*
Erythrocytes (50μl) and hepatocytes were co-incubated with increasing concentrations of proline. The conditions were otherwise identical with those described for Fig. 2. The duration of incubation was 60min. The proline-dependent stimulation of $^{14}CO_2$ production was obtained by subtracting the value obtained without proline. Each point is the mean ± S.E.M. of triplicate determinations.

Requirement of proline oxidase for stimulation of erythrocyte pentose phosphate pathway in co-incubations containing proline

Using hepatocytes from mice with an inherited deficiency of proline oxidase (PRO/Re), we provided evidence that proline stimulation of the erythrocyte pentose phosphate pathway in co-incubations required the conversion of proline into P5C. Co-incubations containing PRO/Re hepatocytes showed no stimulation of $^{14}CO_2$ production from [1-^{14}C]glucose when proline was added, but the addition of 0.3mM-P5C to identical co-incubations produced a greater than 2-fold increase in pentose phosphate-pathway activity (Table 1). Co-incubations containing hepatocytes from normal animals exhibited a greater than 2-fold increase in pentose phosphate-pathway activity when either 5mM-proline or 0.3mM-P5C was added to incubations. These findings indicated that a proline-degradative product was necessary for the interaction to occur.

Metabolic fate of proline in hepatocytes

Although a degradative product of proline appeared to mediate the effect, the mediator may have been a product other than P5C. However, we found that in the presence of rotenone the majority of

Table 1. *Dependence of the proline effect on hepatocyte proline oxidase*
Erythrocytes were co-incubated with hepatocytes from mice deficient in proline oxidase (PRO/Re) or from normal mice (C57/B6). The conditions were otherwise identical with those described in the legend to Fig. 3. Where indicated, proline or P5C were added at 5mM and 0.3mM respectively. Data were normalized to 5mg wet wt. of hepatocytes per incubation and expressed as either the average of duplicate determinations or means ± S.E.M of three determinations. Results of statistical analysis (paired Student's *t* test) are expressed by * ($P < 0.001$) or † (not significant).

Source of hepatocytes for co-incubation	Additions	$^{14}CO_2$ production from [1-^{14}C]glucose (nmol/h per incubation)	
C57/B6	None	6.47 ± 0.44	*
C57/B6	Proline	13.76 ± 0.98	*
C57/B6	P5C	11.16	
PRO/Re	None	5.72 ± 0.34	†
PRO/Re	Proline	6.01 ± 0.06	†
PRO/Re	P5C	15.52	

Table 2. *Metabolic fate of [U-^{14}C]proline in rotenone-treated hepatocytes*

Hepatocytes (3 mg wet wt.) were incubated with [U-^{14}C]proline (5 mM, 5 μCi) for 60 min under the conditions described in the legend to Fig. 2. Proline, 1-pyrroline-5-carboxylate and CO_2 were recovered by the methods described in the text. Glutamate and keto-acids were recovered in previously identified fractions. Data are averages of duplicate determinations or means ± S.E.M. of three determinations.

Proline utilized (c.p.m./ incubation)	$^{14}CO_2$ (c.p.m./ incubation)	Keto-acids (c.p.m./ incubation)	Glutamate (c.p.m./ incubation)	P5C (c.p.m./ incubation)
53358	91	267	1494	31338 ± 389

proline utilized by hepatocytes can be recovered as P5C. To determine the metabolic fate of proline we incubated hepatocytes with [U-^{14}C]proline and quantified labelled proline, CO_2, keto-acids and glutamate, as well as P5C, after incubation. The presence of rotenone markedly inhibited the passage of proline carbon atoms to glutamate and the tricarboxylic acid cycle. With 60 min of incubation little $^{14}CO_2$ was generated from [U-^{14}C]proline (Table 2). Expressed as a function of proline disappearance from the medium, glutamate and keto-acids respectively accounted for only 3% and <1% of proline metabolized. However, large amounts of P5C were produced from proline and nearly 60% of proline metabolized was recovered as P5C. Thus on the basis of proline products recovered, the majority of proline carbon atoms was found in P5C when hepatocytes were treated with rotenone.

Evidence that P5C mediated erythrocyte pentose phosphate-pathway stimulation in co-incubations

Specific validation that P5C mediated the stimulation of erythrocyte pentose phosphate pathway in co-incubations containing proline was provided by experiments with *o*-aminobenzaldehyde, which specifically and covalently binds 1-pyrroline compounds (Johnson & Strecker, 1962; Phang *et al.*, 1973). With increasing concentrations of *o*-aminobenzaldehyde, the stimulation by proline progressively decreased until it was essentially abolished at 2 mM (Fig. 4). Thus *o*-aminobenzaldehyde eliminated proline stimulation of erythrocyte pentose phosphate pathway in co-incubations in a dose-dependent fashion.

Additional experiments indicating that *o*-aminobenzaldehyde produced its inhibitory effect by making P5C unavailable to erythrocytes and not by altering proline metabolism within hepatocytes have already been reported (Phang *et al.*, 1981). Hepatocytes were incubated with or without proline in the absence of the inhibitor. Hepatocytes were removed by centrifugation and the medium was used in subsequent incubations with erythrocytes. The pentose phosphate pathway of erythrocytes was stimulated by the addition of medium previously incubated with hepatocytes and proline, but not by medium incubated with hepatocytes without proline.

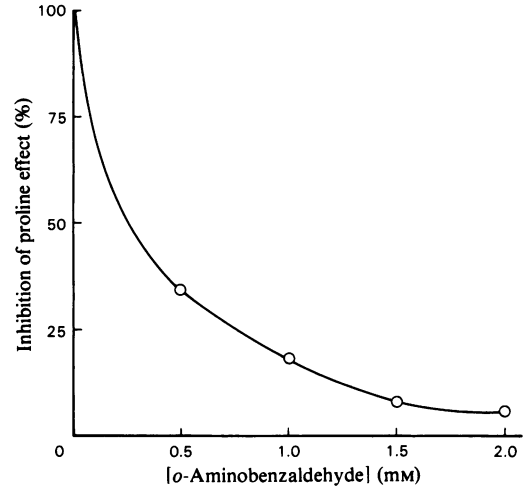


Fig. 4. *Inhibition of the proline effect by increasing concentrations of o-aminobenzaldehyde*

Erythrocytes (50 μl) were co-incubated with hepatocytes under conditions identical with those described in the legend to Fig. 2, except that *o*-aminobenzaldehyde was added. The 100% value was based on the proline-dependent stimulation over controls. Addition of *o*-aminobenzaldehyde to erythrocytes alone and co-incubations without proline had no effect. Data represent averages of three determinations.

The addition of 2 mM-*o*-aminobenzaldehyde to erythrocyte incubations abolished the proline-dependent stimulation in these sequential incubations, indicating that the inhibitor was making P5C unavailable to erythrocyte P5C reductase.

Although rotenone was used routinely in our initial studies to eliminate the hepatocyte tricarboxylic acid cycle as a possible complicating source of $^{14}CO_2$, we later found that it was not required to observe the proline-dependent increase in $^{14}CO_2$ production in co-incubations. We observed that mouse hepatocytes not exposed to rotenone would release P5C into the medium at a rate of 12.0 μmol/30 min per g wet wt. When hepatocyte erythrocyte co-incubations were performed in the absence of rotenone (Fig. 5), we observed a proline-dependent increase in $^{14}CO_2$ production of almost the same

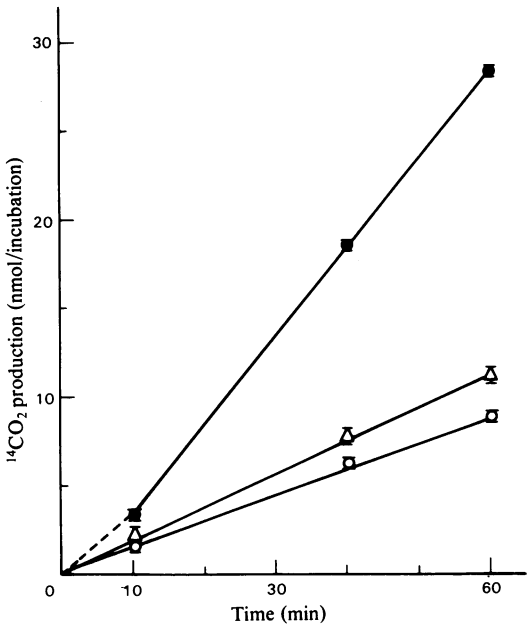


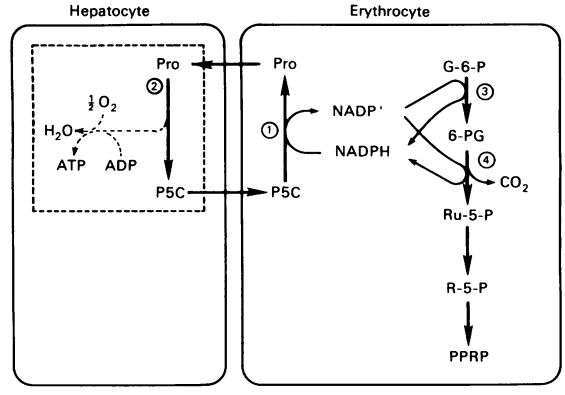
Fig. 5. *Effect of proline on co-incubations without rotenone and inhibition of proline effect by o-aminobenzaldehyde*

Erythrocytes (150 μl) and hepatocytes were co-incubated in the presence (●) and absence (○) of 5 mM-proline, and in the presence of 5 mM-proline and 2 mM-*o*-aminobenzaldehyde (△). The conditions were otherwise identical with those described in the legend to Fig. 2. Data are means ± S.E.M. for at least three determinations.

magnitude as that seen in co-incubations containing rotenone (Fig. 2). Notably, 2 mM-*o*-aminobenzaldehyde added to such incubations containing proline inhibited the proline-mediated effect (74–88%) at all incubation times. Thus the proline-mediated metabolic interaction between hepatocytes and erythrocytes occurs in the absence of rotenone when proline has many metabolic fates (Hensgens *et al.*, 1978). Nevertheless, inhibition of this interaction by *o*-aminobenzaldehyde implicates P5C as the specific mediator of erythrocyte pentose phosphate-pathway stimulation.

Discussion

Our findings suggest that the interconversions of proline and P5C form a metabolic link between hepatocytes and erythrocytes. The demonstration of this metabolic link was possible because of the distinctive metabolic features of each cell type. In erythrocytes P5C stimulates pentose phosphate-pathway activity because P5C reductase oxidizes



Scheme 2. *The proposed intercellular proline cycle between hepatocytes and erythrocytes*

Enzymes are designated as: (1) P5C reductase; (2) proline oxidase; (3) glucose 6-phosphate dehydrogenase; (4) 6-phosphogluconate dehydrogenase. Abbreviations used: G-6-P, glucose 6-phosphate; 6-PG, 6-phosphogluconate; Ru-5-P, ribulose 5-phosphate; PPRP, 5-phosphoribosyl pyrophosphate; R-5-P, ribose 5-phosphate.

NADPH while converting P5C into proline (Yeh & Phang, 1980). Proline is without effect in erythrocytes because they have no proline oxidase and thus cannot convert proline into P5C. On the other hand, hepatocytes have both proline oxidase and P5C reductase activity, but their pentose phosphate-pathway activity is unaffected by either P5C or proline. Inhibition of hepatocyte P5C reductase *in situ* by ATP or $NADP^+$ may be a possible explanation (Smith & Greenberg, 1957; Peisach & Strecker, 1962). Alternatively, the contribution of P5C to oxidizing potential in hepatocytes may be negligible compared with that from other sources. Nevertheless, hepatocytes produce P5C from proline and more importantly release P5C into the incubation medium.

On the basis of our findings we propose that an intercellular proline cycle is the basis for the proline effect in co-incubations (Scheme 2). Proline is converted into P5C by proline oxidase in hepatocytes and P5C is transferred from hepatocytes to erythrocytes. P5C is then converted back into proline by P5C reductase within erythrocytes, with concomitant oxidation of NADPH and stimulation of the pentose pathway in erythrocytes. Finally, proline is released into the medium by erythrocytes and is available again to re-enter hepatocytes and repeat the cycle.

Presumably P5C mediates pentose phosphate-pathway stimulation in erythrocytes by increasing $NADP^+$ availability, i.e. turnover, and perhaps by increasing $[NADP^+]/[NADPH]$ ratios (Yeh &

Phang, 1980). Regulation of the pentose phosphate pathway can occur in several ways. In liver, high-carbohydrate diets will produce adaptive increases in glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Tepperman & Tepperman, 1958; Fitch *et al.*, 1959; Fitch & Chaikoff, 1960). In cell cultures the flux through the pentose phosphate pathway can be affected by the type and concentration of carbohydrate in the medium (Reitzer *et al.*, 1980). However, finer regulation of this pathway most likely occurs at the level of glucose 6-phosphate dehydrogenase, the site of entry and the only irreversible step in the pentose phosphate pathway. At this site both [$NADP^+$] and [$NADP^+$]/[NADPH] ratios may have a regulatory function. Complete inhibition of glucose 6-phosphate dehydrogenase occurs at [$NADP^+$]/[NADPH] ratios below 0.12 (Krebs & Eggleston, 1974). Since the [free $NADP^+$]/[free NADPH] ratio in erythrocytes and in rat liver has been estimated to be 0.01, glucose 6-phosphate dehydrogenase may be completely inhibited *in vivo* (Veech *et al.*, 1969; Krebs & Eggleston, 1974). Thus special mechanisms for removing the inhibition of glucose 6-phosphate dehydrogenase activity may be essential for regulation of the pentose phosphate pathway. Krebs & Eggleston (1974) have proposed that oxidized glutathione and a dialysable cofactor may provide such a mechanism for removing the inhibition of glucose 6-phosphate dehydrogenase *in situ*. In this regard, P5C reductase provides an alternative mechanism for oxidizing NADPH and stimulating pentose phosphate-pathway activity by removing NADPH inhibition of glucose 6-phosphate dehydrogenase (Yeh & Phang, 1981). Indeed P5C has been demonstrated to be a potent stimulator of the pentose phosphate pathway in erythrocytes (Yeh & Phang, 1980) as well as fibroblasts (Phang *et al.*, 1979).

There is substantial evidence that the proposed metabolic interaction between hepatocytes and erythrocytes results in stimulation of erythrocyte pentose phosphate pathway, but a physiological role for this stimulation has not been established. Nevertheless, recent studies have emphasized the importance of the oxidative arm of the pentose phosphate pathway in pentose phosphate and nucleic acid synthesis (Reitzer *et al.*, 1980). In quiescent cells undergoing mitogenic stimulation (Smith & Buchanan, 1979) or cells with carbohydrate limitation (Reitzer *et al.*, 1980), these latter authors suggest that the production of pentose is the critical function of the pentose phosphate pathway. Although the generation of NADPH has been considered the major role of the pentose phosphate pathway's oxidative arm in erythrocytes, it may also be an important source of ribose 5-phosphate for 5-phosphoribosyl pyrophosphate synthesis. Erythrocytes require 5-phosphoribosyl-pyrophosphate for purine salvage and they lack pathways for purine biosynthesis *de novo* (Murray, 1971). Furthermore, the demand for 5-phosphoribosyl pyrophosphate synthesis within erythrocytes may exceed its own requirements for nucleotides because erythrocytes may be involved in the uptake, processing and delivery of purines from hepatic stores to peripheral tissues (Pritchard *et al.*, 1970; Konishi & Ichihara, 1979). Thus a possible physiological consequence of the intercellular proline cycle may be enhancement of erythrocyte 5-phosphoribosyl pyrophosphate production to augment the uptake and transfer of purines to peripheral tissue.

Previous investigators suggested that erythrocytes play a role in the inter-organ transport of amino acids (Elwyn *et al.*, 1972; Felig *et al.*, 1973). However, we now propose that erythrocytes may participate in the intercellular cycling of an amino acid and its product. Since erythrocytes come into close proximity to certain cells, e.g. hepatocytes, metabolic cycling may occur between two cell types *in situ* and may not be detected by studies based on arterial–venous differences in amino acids. If the metabolic interaction proposed occurs as a cycle, one would not expect any net change in proline or P5C concentration within the organ involved. Considering the activity of P5C reductase in erythrocytes and their ability to rapidly convert P5C into proline, it is not surprising that P5C has been undetectable in peripheral venous plasma (Goodman *et al.*, 1974). However, detectable concentrations of P5C in specific organ effluents remains a possibility that requires further investigation.

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