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CREATINE AND CREATININE TRANSPORT IN OLD AND YOUNG HUMAN RED BLOOD CELLS

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Summary

The time course of creatine influx or efflux as measured in populations of red cells or red cell ghosts with normal age distribution does not follow simple two-compartment kinetics. This suggests that the contributions of individual cells to transport as measured in the populations as a whole are not uniform. In agreement with this inference, fractionation of red cell populations with respect to cell age shows that transport in young cells is considerably faster than in old cells.

The dependence of creatine transport on creatine concentration in the medium follows an equation that can be interpreted to represent a superimposition of a saturable component (apparent K_m = 0.02 mM) and another component that cannot be saturated up to a creatine concentration of 5.0 mM. In contrast to the non-saturable component, the saturable component depends on the energy metabolism of the cell and can be inhibited by β-guanidinopropionic acid and the proteolytic enzyme pronase. This latter finding suggests that the saturable component represents active transport that is mediated by a transport protein. The non-saturable component is little, if at all, dependent on cell age while the saturable component is higher in young cells than in old cells. Phloretin inhibits both components of creatine flux, but the maximal inhibition that can be achieved at high concentration is only 70—80%.

Under the experimental conditions used for the study of creatine transport, creatinine equilibration between cells and medium follows the kinetics expected for a steady-state two-compartment system. Creatinine flux is proportional to

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Abbreviations: APMB, 2-(4′-aminophenyl)-6-methylbenzenethiazol-3′,7-disulfonic acid; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid; NMG, *N*-methylguanidine; GAA, guanidinoacetic acid; Hepes, *N*-2′-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Hb, hemoglobin.

creatine concentration over the concentration range studied (up to 5 mM). It cannot be inhibited by β-guanidinopropionic acid or pronase.

Introduction

In contrast to muscle cells, adult red blood cells contain little, if any, creatine phosphate [1,2]. This seems to reflect the fact that in red cells sudden changes of energy requirements are unlikely to occur and that for this reason no high-energy phosphate other than nucleotide triphosphates is needed. Nevertheless, red cells contain some creatine. The creatine concentration in young cells is higher than in old cells [3—5] such that the average creatine concentration represents a measure of the average age of the cells in a population (Refs. 3 and 6; however, see also Ref. 7). At least in the younger red cells the intracellular concentration is higher than the concentration in the plasma [2]. The concentration difference is maintained in spite of the fact that the red cell membrane is permeable to creatine [8—10]. This suggests the involvement of active transport.

The experiments presented in this paper deal with creatine transport in old and young red blood cells and the effects of chemical and enzymatic modification of the cell membrane on the transport process. They aim at clarifying two questions which need to be answered before more systematic studies of the molecular mechanism of creatine transport in red blood cells can be initiated.

The first question concerns the time course of equilibration of creatine in red cell populations with a normal age distribution. Deviations from a semilogarithmic relationship would indicate kinetics that are different from those of a simple steady-state two-compartment system and point to a heterogeneity of the cell population with respect to creatine transport. As will be shown below, such deviations are actually observed. This led immediately to the question as to whether or not these deviations can be correlated with differences of cell age in the population. For this reason we separated red cell populations into fractions with different age distribution and studied the rate of transport in these fractions separately.

The second question pertains to the kinetics of transport in the individual cells: does the creatine transport take place via one single pathway or are several parallel pathways involved, e.g., one pathway for active transport and another for passive transport? To answer this question we first studied the dependence of creatine flux on creatine concentration and looked for saturable and non-saturable components of the transport process. We confirmed previous observations of the existence of two different components [9] and tried to find suitable inhibitors (chemicals and proteolytic enzymes) that could be used for further verification of the inferences drawn from the flux vs. concentration relationship. Finally, we modified the energy metabolism of the cells in order to differentiate between pathways for active and passive transport.

Our results suggest that creatine is transported via two parallel pathways. One of these is saturable at low creatine concentrations and can be inhibited by metabolic depletion, by the addition of the substrate analogue, β-guanidinopropionic acid, and by treatment of the cell membrane with the proteolytic

enzyme pronase. This is compatible with the assumption that this pathway is responsible for active transport and that a transport protein is involved. The other pathway showed no sign of saturation over the concentration range studied and could not be inhibited by β-guanidinopropionic acid or pronase. This suggests transport by diffusion or by a low-affinity transport system without participation of cell metabolism.

The zwitterionic creatine is removed from the body after conversion into the electrically neutral creatinine molecule ('metabolic garbage' [11]). We were interested, therefore, to compare creatine transport with that of creatinine. It was observed that creatinine equilibrium exchange followed two-compartment kinetics and was linearly related to creatinine concentration with no indication of the existence of a saturable component. Neither β-guanidinopropionic acid nor pronase had any influence. Apparently, creatinine is transported as such by diffusion or after combination with a low-affinity transport system. Since hexanol was found to have no effect on creatinine transport we suspect that diffusion through the lipid phase is not involved.

Materials and Methods

Efflux measurements. Human blood (type 0; Rh^+) was stored in acid/citrate/dextrose buffer and used within 5 days after withdrawal. After removal of plasma and buffy coat the cells were washed three times with a medium containing 121 mM NaCl, 10 mM KCl, 20 mM sodium phosphate, pH 7.4. Resealed ghosts were prepared essentially as described previously [12]. They were hemolysed at a cell : medium ratio of 1 : 20 in a medium containing 4 mM $MgSO_4$ and 1.3 mM acetic acid. 5 min after hemolysis, sufficient KCl, NaCl, glycylglycine (or the other buffers mentioned below) and $MgCl_2$ were added to obtain final concentrations of 131, 10, 20 and 2.0 mM, respectively. Creatine or creatinine were added at the concentrations desired. The pH was 7.2. 10 min later, the ghosts were pelleted at 20 000 rev./min for 5 min, resuspended in their own supernatant at 50% hematocrit, and sufficient [1-^{14}C]creatine or [*carbonyl*-^{14}C]creatinine was added to give approx. 0.25—0.5 μCi/ml. Up to this point the temperature was 0°C. For resealing, the ghosts were incubated at 37°C for 60 min. At the end of the resealing period, the ghosts were washed twice with ice-cold 'flux medium'.

Efflux was initiated by suspending 0.2 ml of labeled ghosts in 9.8 ml of the 'flux medium' (composition: 121 mM NaCl, 10 mM KCl and 20 mM buffer, either phosphate or glycylglycine or Hepes, pH 7.4). The ghosts density was usually 2 vol%, the temperature 37°C, the pH 7.4. At suitable time intervals, aliquots of the suspension were centrifuged, the supernatants were collected, mixed with an equal volume of 21% trichloroacetic acid and used for the determination of radioactivity in a liquid scintillation counter. The radioactivity at infinite time was determined by mixing equal volumes of suspension and 21% trichloroacetic acid, centrifuging and counting an aliquot of the supernatant.

Efflux from intact red cells was measured as described in the legend to Fig. 1.

Influx measurements. The influx was initiated by suspending a measured volume of washed cells or resealed ghosts in one of the flux media described

above. These media contained approx. 0.1—0.25 μCi/ml of [^{14}C]creatine or [*carbonyl*-^{14}C]creatinine (cell or ghost density 10 vol%, pH 7.4, 37°C). At suitable time intervals, aliquots of the suspensions were withdrawn, mixed with a 10-fold volume of an ice-cold solution containing 121 mM NaCl, 10 mM KCl, 20 mM sodium phosphate, pH 7.4, centrifuged and washed once in the same ice-cold solution. After dissolution in distilled water and precipitation of the proteins by trichloroacetic acid, aliquots of the supernatants were mixed with liquid scintillator and counted. For the determination of radioactivity at infinite time, an aliquot of the suspension was precipitated with trichloroacetic acid without prior centrifugation and washing, and the supernatant was used for liquid scintillation counting as described above.

The actions of inhibitors on creatine and creatinine movements were determined by adding the various agents to the flux medium. The pH was always adjusted to 7.4. Trypsin treatment of the red cells was carried out at 37°C for 60 min at a hematocrit of 20% in the medium described above. The action of the enzyme was stopped by adding soy bean trypsin inhibitor and subsequent incubation for 10 min at 37°C. The cells were then washed twice with the medium described, but without trypsin inhibitor. Treatment of the red cells with other enzymes was performed under the same conditions as treatment with trypsin except that at the end of the incubation period the cells were centrifuged and washed once with a medium containing 0.5% bovine serum albumin and two additional times with the same medium but without bovine serum albumin. In some of the experiments the enzymes were simply dissolved in the flux medium.

The separation of human red cells according to age was carried out on discontinuous density gradients of Stractan II, essentially as described by Corash et al. [13]. After centrifugation, the segregated bands of red cells of different density were collected and resuspended in the usual washing solution. They were washed twice in Stractan-free medium and then used for the measurement of creatine flux.

Chemicals and enzymes were obtained from the following sources: APMB, a gift from Professor Petersen, Beyer, Leverkusen; H_2DIDS, synthesized by Professor Fasold, as described previously [14]; persantin, a gift from Professor Deuticke, Aachen; phlorizin, Roth KG, Karlsruhe; hexanol, γ-guanidinobutyric acid, β-guanidinopropionic acid, 4-guanidinobutyric acid, mineral oil, Stractan II (an arabinose-galactan polysaccharide), Sigma Chemical Co.; trypsin, pronase, Merck AG, Darmstadt; chymotrypsin, soy bean trypsin inhibitor, Boehringer-Mannheim. ATP, creatine, creatinine, amberlite MB-3, Serva, Heidelberg; [1-^{14}C]creatine, [*carbonyl*-^{14}C]creatinine, The Radiochemical Centre, Amersham. ^{59}Fe-labeled red cells were obtained from the Department of Hematology of the University of Frankfurt. We thank Dr. Rogge and Professor Martin for their support.

Results

Time course of creatine and creatinine movements

The time course of equilibration across the red cell membrane of many solutes, including K^+ [15,16], anions [17] and erythritol [18] can be repre-

sented by a single exponential, and thus follows the kinetics expected for equilibration in a steady-state two-compartment system. However, an analysis of kinetic data in terms of a two-compartment system is only permissible if all cells in the population have equal volume/surface ratios and equal permeabilities (per cm^2). This does, of course, not apply to the naturally occurring erythrocyte populations where at least the volume/surface ratios show a statistical distribution. A mathematical analysis of the effects of a Gaussian distribution of the rate-determining parameters among the individual cells of a population on the time course of equilibration under steady-state conditions has been performed by Sheppard and Householder [19]. It was concluded that the deviations from a single exponential that one can expect in red cell populations are rather small for most solutes. Therefore, the single rate constant derived from self-exchange experiments usually represents a reasonable measure of the average behavior of the cells in the population [19].

Creatinine self exchange as measured under steady-state conditions in red cell ghosts and intact red cells (Fig. 1b) closely follows two-compartment kinetics. It is possible, therefore, to calculate rate constants that are reasonably representative for the average behavior of the cells in the population.

In contrast, the time course of equilibration of creatine in ghosts or intact red cells (Fig. 1a) does not follow these kinetics. After an initial fast phase the curves tend to approach a straight line. However, this does not imply that the process is simply diphasic. During the limited time period of experimental observation, the system is too far from equilibrium to permit an extrapolation

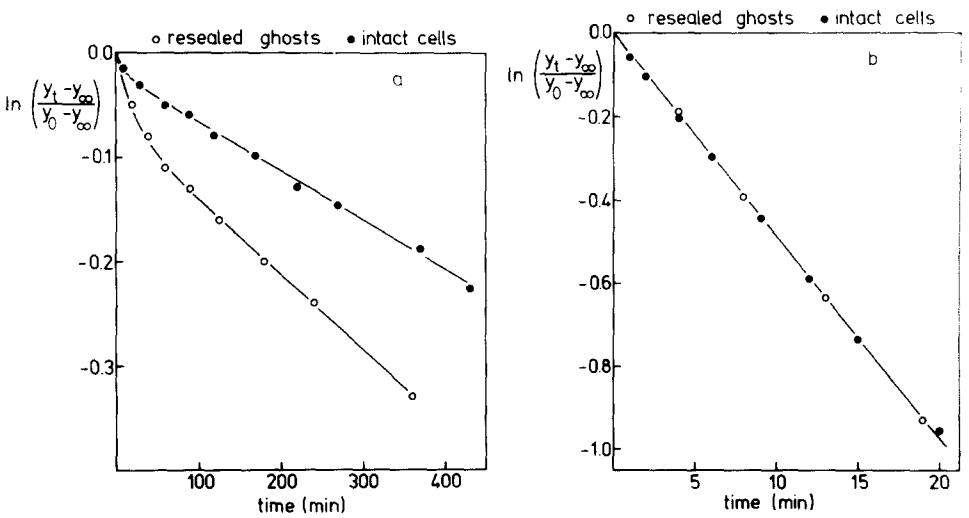

Fig. 1. Semilogarithmic representation of creatine (a) and creatinine (b) efflux from intact human red blood cells and resealed red blood cell ghosts. For labeling with [^{14}C]creatine the intact cells were incubated at 37° C for 12 h in a medium containing 0.1 mM creatine and [^{14}C]creatine (1.25 μCi/ml) at 50% hematocrit. Efflux was measured after resuspension in a creatine-free medium. In the experiments with resealed ghosts internal and external concentrations of creatine are equal and amount to 0.5 mM. For labeling with [^{14}C]creatinine intact cells were incubated at 37° C for 2 h in a medium containing 0.5 mM creatinine and [^{14}C]creatinine (1.25 μCi/ml) at 50% hematocrit. Internal concentration of creatinine was 0.5 mM and equal to the concentration in the flux medium for both intact cells and resealed ghosts.

to longer time periods, and experiments which did extend over longer time periods (up to 25 h) have shown that such extrapolation is indeed not possible (not documented).

The uptake of creatine and creatinine follows the same pattern as the release: deviations from a single exponential for the former and good agreement for the latter (not shown).

Dependence of creatine transport on red cell age

Deviations from a single exponential suggest that the behavior of the individual cells (or ghosts) in the populations is not uniform. This is confirmed by the experiment in Fig. 2 which demonstrates the time course of uptake of creatine in four fractions of red cells which had been separated according to age by centrifugation on a discontinous density gradient. The inset shows that the various fractions obtained on the gradient indeed contain cells with different age distributions and the curves indicate that the rate of creatine uptake decreases with increasing proportion of old cells in the fractions. The difference of transport rates in young and old cells is higher at 0.1 mM creatine in the medium than at 0.5 mM (see below).

Although the differences of transport rates are clearly apparent from the experiment depicted in Fig. 2, it should be noted that at least two additional factors contribute to the deviations from the steady-state two-compartment kinetics. Firstly, the younger cells are known to contain more creatine than older cells [3—5]. Since the rate of equilibration not only depends on the rate

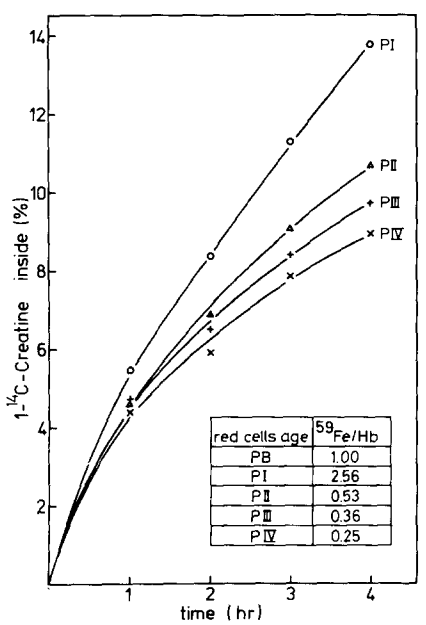

Fig. 2. Uptake of [^{14}C]creatine by red cell populations with different age distribution. Creatine influx was measured at 0.5 mM creatine in the flux medium. PI—PIV, fractions with increasing cell age; PB, population before separation. The efficiency of the separation procedure was checked by the use of ^{59}Fe-labeled red blood cells. The table shows the ratio of ^{59}Fe/Hb in the various fractions in multiples of the average observed in the population before separation.

of transport but also on the size of the compartment, we may conclude that the observed differences of transport rates between young and old cells represent an underestimate. Secondly, the creatine distribution between cells and medium is unlikely to be in the steady state. However, from the work of Syllm-Rapoport et al. [9] it is apparent that the creatine exchange as measured with the ^{14}C-labeled compound is considerably faster than the net movements as determined by chemical analysis of the creatine. Thus the net movements are not likely to be of major significance for the observed kinetics of isotope equilibration. This inference is supported by our finding that in ghost populations, where the unlabeled creatine is certainly at equilibrium between cell interior and medium, the deviations from two-compartment kinetics are also apparent (Fig. 1a).

Definition of a measure for the transport rate in cell populations with normal age distribution

The observation that different cells in the population transport creatine at different rates makes it difficult to define a measure of transport for the cell population as a whole. At short times the influence of young cells with high transport rates tends to predominate, whilst after long times, the contribution of old cells with low transport rates becomes more significant. Thus, strictly speaking, an average rate of transport is not meaningful. Nevertheless, if one wants to compare the effects on transport of different concentrations of the substrates or inhibitors, it is necessary to find some quantity that represents a measure of the transport in the population. Although a mathematical treatment of the situation described seems feasible, such treatment would be difficult to apply to actual observations since the choice of the many parameter values that are necessary to describe the age dependency is too arbitrary to allow a unique fit. For this reason, we averaged over the contributions of cells of various age groups: we followed the transport for the first 3—4 h and formed the average of the hourly increments measured during that time. These increments show a continuous decrease with time, with a certain tendency to become constant after about 6—7 h (Fig. 3). The absolute values of the calculated averages are not meaningful. However, they are useful for the calculation of ratios between

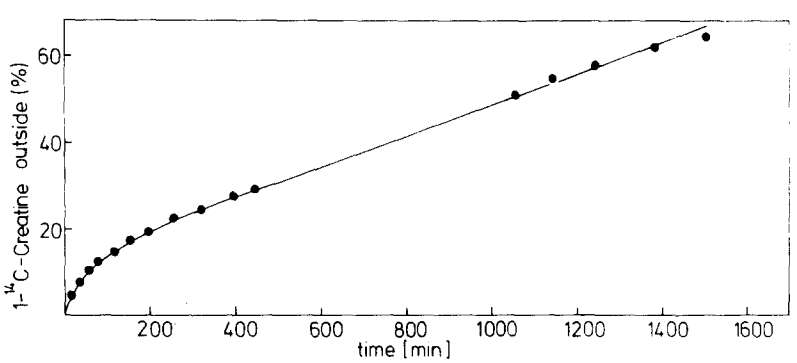

Fig. 3. Creatine efflux from resealed ghosts. Experimental conditions as described in Fig. 1.

transport in controls and in cells which had been exposed to a range of concentrations of substrates or inhibitors.

Concentration-dependence of creatine and creatinine transport

Creatine uptake as a function of creatine concentration follows a pattern that can be described by a superimposition of a saturable and a non-saturable component (Fig. 4a). This can be expressed by the empirical equation:

$$\text{transport} = V\frac{[C_r]}{K + [C_r]} + A \cdot [C_r]$$

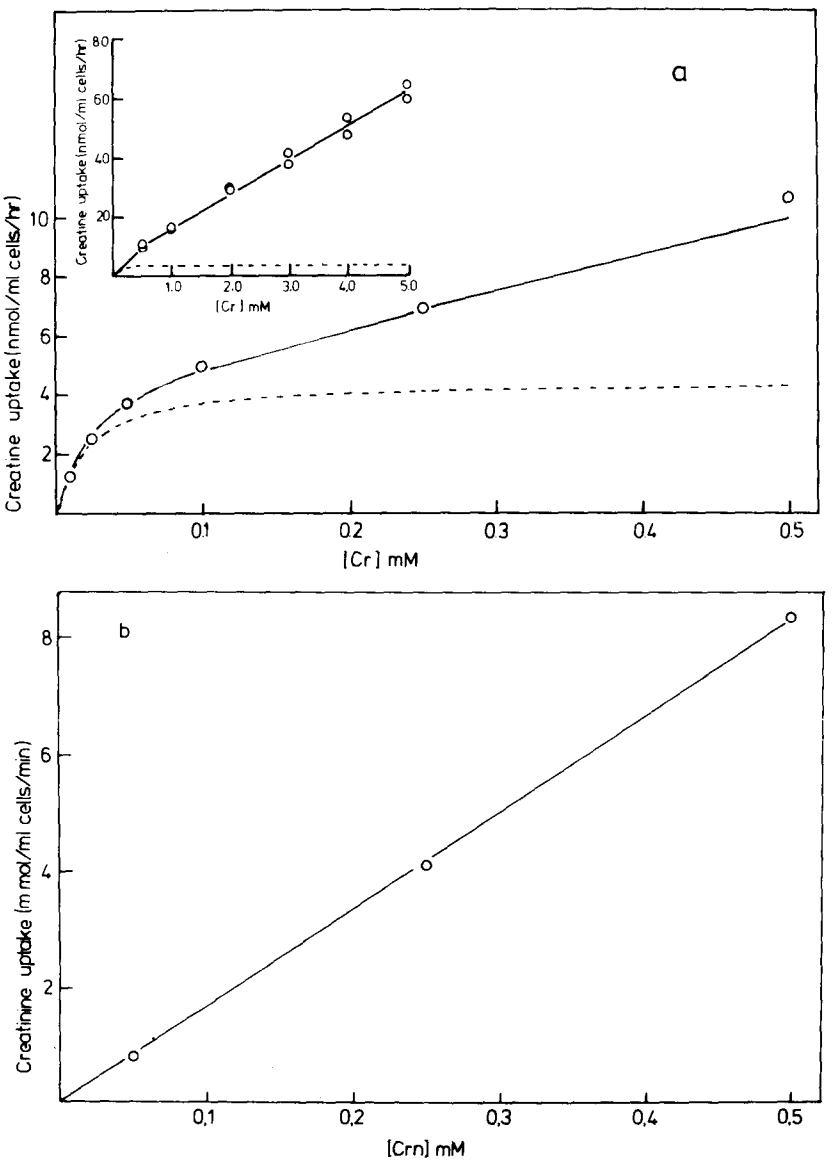

Fig. 4. The dependence of creatine (a) and creatinine (b) uptake into red blood cells as a function of creatine (Cr) and creatinine (Crn) concentration, respectively.

$V = 4.5$ nmol · ml^{-1} · h^{-1}, $K = 0.021$ mM and $A = 11.54$ h^{-1}, if the concentrations are inserted in units of mM.

In contrast to creatine transport, the dependence of creatinine transport on creatinine concentration can be represented by a straight line which passes through the origin (Fig. 4b), suggesting the absence of an easily saturable transport system for this compound.

Experiments with inhibitors

Table I lists the inhibitors which have been used and the degrees of inhibition observed. Two of the inhibitors proved to be of major usefulness: phloretin and β-guanidinopropionic acid. In influx experiments phloretin produces maximally approx. 70% inhibition for creatine and approx. 80% inhibition for creatine (Fig. 5a and c), β-guanidinopropionic acid has no effect on creatinine transport and causes approx. 45% inhibition of creatine transport (Fig. 5b). The effects of the two agents on creatine transport are not additive. Both agents produce inhibition of influx in intact cells and ghosts. In experiments with ghosts efflux was not inhibited.

The effectiveness of β-guanidinopropionic acid differs in young and old red cells. When present at a nearly maximally inhibitory excess over creatine, the percentage inhibition in a preparation of young cells was approx. 72%, in a preparation of older cells approx. 50%. Interestingly enough, the rate of uptake in the maximally inhibited cells is nearly the same in the two preparations, suggesting that β-guanidinopropionic acid only inhibits the increment of creatine transport above an age-independent level (Fig. 6).

Experiments with proteolytic enzymes

(a) Pronase produces a dual effect on creatine influx into intact cells. If the

TABLE I

THE EFFECT OF VARIOUS AGENTS ON CREATINE AND CREATININE INFLUX INTO HUMAN RED CELLS

Inhibitors used were dissolved in the flux medium for measuring creatine and creatinine influx in intact red cells. —, not measured.

Inhibitor	mM	Inhibition (%)	
		Creatine	Creatinine
APMB	1.00	0	—
H_2DIDS	0.01—0.12	*	0
β-guanidinopropionic acid	1.00	30	0
γ-guanidinobutyric acid	1.00	0	—
NMG	1.00	0	—
GAA	1.00	0	—
4-guanidinobutyric acid	1.00	0	—
Sarcosine	1.00	0	—
Hexanol	8.00	20	0
Persantin	0.02	45	—
Phlorizin	1.00	30	—
Phloretin	1.00	80	70

* Inhibition is variable; usually there is no effect, but sometimes up to 20% inhibition may be observed.

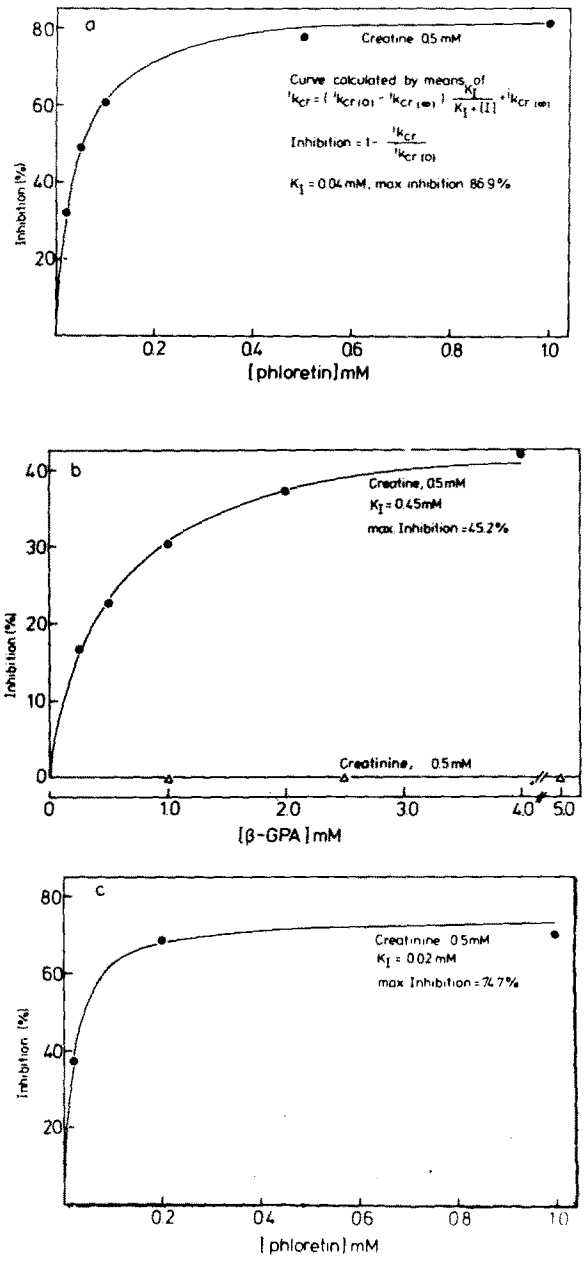

Fig. 5. Inhibition by phloretin and β-guanidinopropionic acid (β-GPA) of creatine and creatinine uptake into intact red cells. Creatine and creatinine concentrations in all experiments 0.5 mM. (a) Percent inhibition produced by phloretin of creatine influx vs. phloretin concentration; (b) percent inhibition by β-guanidinopropionic acid of creatine and creatinine influx vs. β-guanidinopropionic acid concentration; (c) percent inhibition by phloretin of creatinine influx vs. phloretin concentration. ● and △, experimental data. The curves pertaining to the data which are represented by the filled circles have been calculated according to the equation indicated in Fig. 5a. In this equation: $^{i}k_{Cr}$, $^{i}k_{Cr(0)}$, $^{i}k_{Cr(\infty)}$ and K_I represent, respectively, the rate constants at the inhibitor concentrations [I], zero, and infinity, and the constant for half-maximal inhibition.

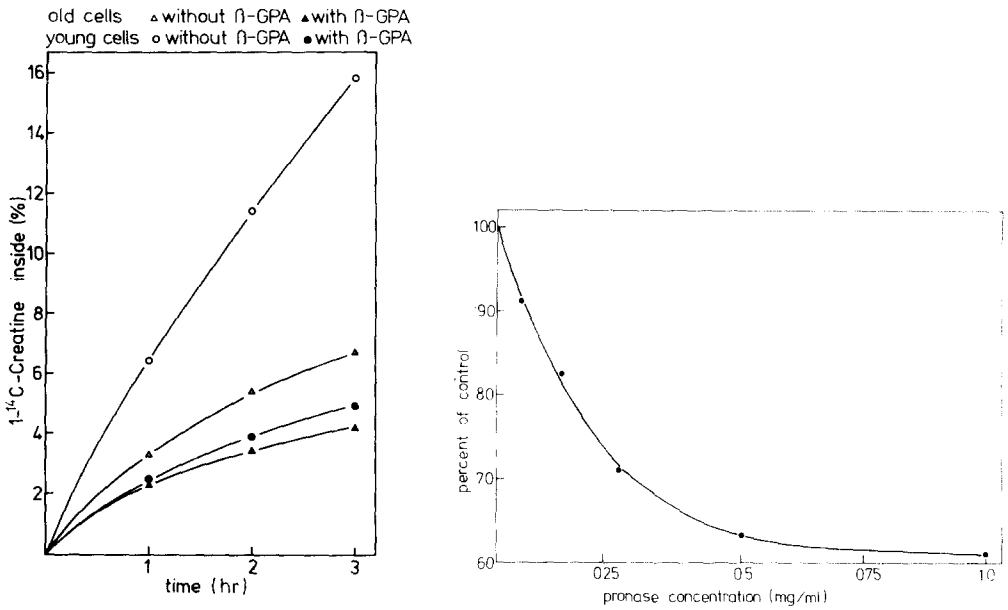

Fig. 6. Inhibitory action of β-guanidinopropionic acid (β-GPA) on cell populations with different age distributions. Creatine influx was measured at 0.1 mM creatine. ○ and ●, population of younger cells; ▵ and ▲, older cells. The two populations correspond to populations PI and PIV in Fig. 2. The β-guanidinopropionic acid concentration was 3.0 mM.

Fig. 7. Effect of pronase on creatine influx into intact red cells. The blood cells were pretreated with various concentrations of pronase at 37°C for 1.0 h; prior to the flux measurements, the enzyme was removed by washing with a buffer containing 0.5% bovine serum albumin. Ordinate, influx as a percent of untreated control; abscissa, pronase concentration (mg/ml). The creatine concentration was 0.5 mM.

cells are exposed to concentrations up to approx. 1 mg/ml for 1 h at 37°C prior to removal of the enzyme and the flux measurements, there is increasing inhibition, up to a plateau at approx. 40% (Fig. 7). After treatment with higher concentrations an enhancement of transport is observed which reaches approx. 140% at 2 mg/ml, the highest concentration studied (not documented).

(b) Trypsin treatment (50 μg/ml, 1 h, 37°C) had no effect on creatine influx into intact red cells. However, ghosts made from trypsinized intact cells showed an enhancement of creatine influx as compared to ghosts made from untreated red cells. This enhancement was quite substantial and amounted up to approx. 200% of the control (Fig. 8). This enhancement does not represent increased leakage. This is indicated by the observation that the increment of creatine efflux in resealed ghosts that have been derived from trypsinized red cells can be nearly completely abolished by the presence of trypsin in the medium into which the efflux takes place (Fig. 9). This effect is also seen with chymotrypsin in the medium but not with papain or bovine serum albumin. If trypsin is added to the medium together with trypsin inhibitor, the described effect does not take place. This cannot be related to the action of an excess of the trypsin inhibitor on the membrane since the inhibitor added alone to the ghosts made from trypsinized or untreated red cells has no effect (Fig. 9).

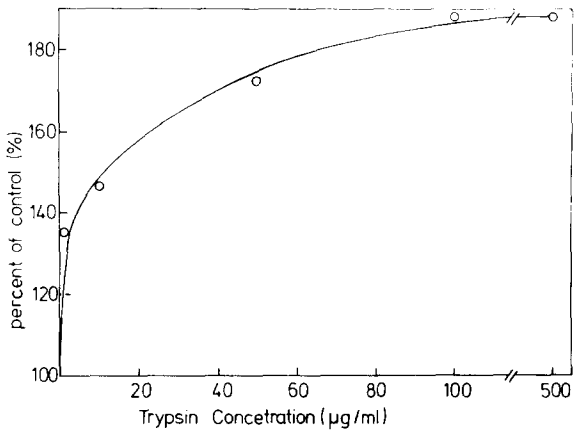

Fig. 8. Creatine transport in resealed ghosts made from trypsinized intact cells. Ordinate, efflux as a percent of untreated control; abscissa, trypsin concentration (μg/ml). Internal and external creatine concentrations are 0.5 mM.

If ghosts are made from red cells which have been treated with a pronase concentration that reduces influx into the intact cells, creatine efflux is enhanced, as described for ghosts made from trypsinized red cells. This enhancement can also be inhibited by the addition of trypsin to the medium into which the creatine efflux takes place.

Neither pronase nor trypsin had any detectable effect on creatinine efflux from resealed ghosts made from the enzymatically treated red cells (Fig. 10a and b).

Effects of metabolic substrates

In a number of experiments we studied the influence of substrates of red cell metabolism on the creatine uptake by intact cells. For this purpose, red cell

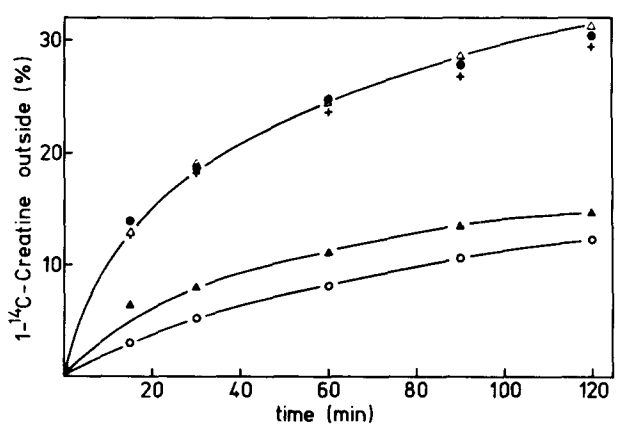

Fig. 9. Creatine efflux from resealed ghosts made from trypsinized (200 μg/ml) and untreated red cells (control). Internal and external concentrations of creatine are 0.5 mM. Control, ghosts made from untreated red cells (○). Ghosts made from trypsin pretreated red cells. No addition to the flux medium (△). Flux medium containing: ▲, 100 μg/ml trypsin; +, 200 μg/ml trypsin inhibitor; ●, 100 μg/ml trypsin plus 200 μg/ml trypsin inhibitor. For explanation see text.

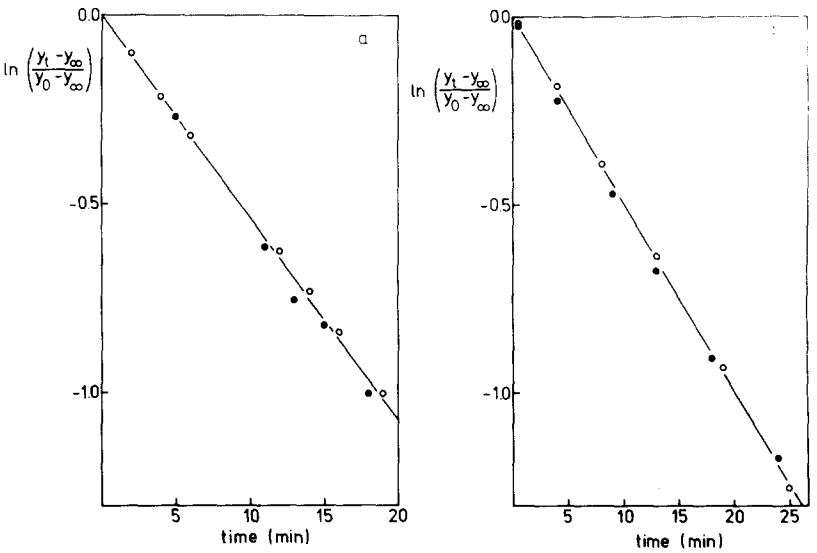

Fig. 10. Semilogarithmic representation of creatinine efflux from resealed red cell ghosts. The internal and external concentrations of creatinine are 0.5 mM. (a) ○, control; ●, pretreatment with 500 μg/ml pronase. (b) ○, control; ●, pretreatment with 50 μg/ml trypsin.

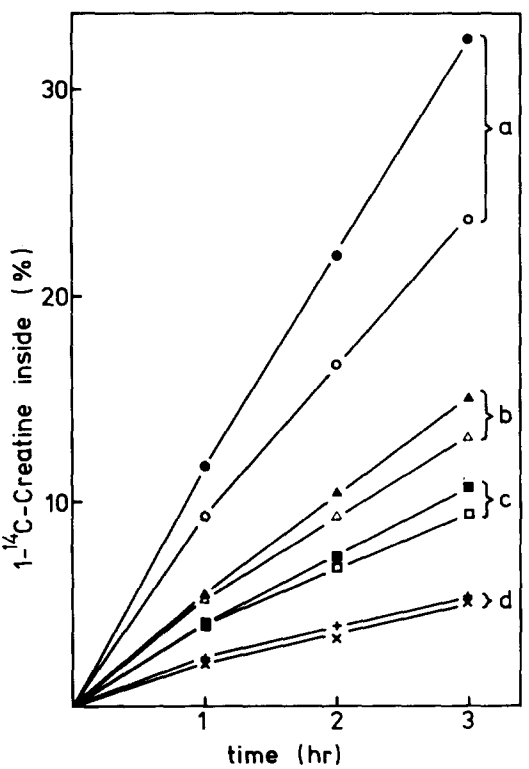

Fig. 11. Creatine influx into metabolizing and substrate-depleted red cells. Creatine concentrations: (a) 0.01 mM (●, metabolizing cells; ○, depleted cells); (b) 0.05 mM (▲, metabolizing cells; △, depleted cells); (c) 0.1 mM (■, metabolizing cells; □, depleted cells); (d) 0.5 mM (X, metabolizing cells; +, depleted cells). For details see text.

suspensions were subdivided into two portions. One was incubated at 37°C in the absence of substrates for 4.5 h, the other was kept on ice for 3 h and was subsequently incubated for 1.5 h at 37°C in the presence of 2 mM adenosine and 10 mM inosine, pH 7.4. Subsequently, both batches of cells were washed in substrate-free media and resuspended in substrate-free media for the measurement of creatine influx. The effect observed after the different types of pretreatment depends on the creatine concentration at which the influx is measured. At high creatine concentrations (greater than 0.1 mM) there is little if any difference between depleted and metabolizing red cells. However, at lower concentrations (less than 0.1 mM) the depleted red cells take up the creatine at a rate which is approx. 30—40% lower than that of the metabolizing cells (Fig. 11).

In another series of experiments we incorporated into red cell ghosts ATP, creatine phosphate or creatine phosphate + ATP, or no substrate. The creatine concentration inside was equal to the creatine concentration in the medium and amounted to 0.1 mM. The effects of the various substances on the rate of creatine uptake by the ghosts were not uniform. In most of the experiments, creatine influx was enhanced when the ghosts contained either one of the two substrates or both of them, in the other experiments the substrates produced no detectable effect.

Discussion

As has been shown in Fig. 4a, a plot of the transport rates vs. the creatine concentration leads to a relationship that can be interpreted as the superimposition of a saturable component with an apparent K_m of approx. 0.021 mM and another component that shows no sign of saturation up to 5.0 mM. The K_m value indicated above is lower than an earlier estimate of Syllm-Rapoport et al. [9]. Moreover, in contrast to these authors we were unable to determine a K_m value for the other process. However, it should be recalled that all numerical values of the parameters of the creatine transport are subject to considerable uncertainties since they involve the use of rather arbitrarily defined measures of the transport rates.

It is reasonable to assume that the process with the low K_m value represents active transport whilst the other refers to diffusion, either of the uncombined creatine molecule or via a carrier with a very high K_m value. This idea of the existence of two pathways is corroborated by several observations.

Firstly, it has been shown that at creatine concentrations of 0.5 mM, the inhibitor β-guanidinopropionic acid (which is known to inhibit creatine transport in muscle; see Refs. 20 and 21) produces maximally no more than 45—50% inhibition. This corresponds closely to the saturable component of creatine transport that one can expect to measure at that creatine concentration.

Secondly, the influx of creatine into metabolically active red cells is much faster than into metabolically depleted cells. Although there was a considerable variability from one batch to another, in many (although not in all) experiments with red cell ghosts we found that creatine phosphate, ATP or both enhance the uptake of creatine as compared to ghosts which contain none of

these substrates. Since red cells contain only little creatine kinase [22], it is unlikely that the increased rate of uptake into metabolically active red cells or high-energy phosphate-containing red cell ghosts can be accounted for by a phosphorylation of the entering creatine and storage in the form of creatine phosphate. It is likely, therefore, that the extra uptake observed under metabolically favorable conditions refers to the saturable component of creatine influx and represents active transport.

This conclusion is supported by the finding that the facilitating effect of enhancing the cell metabolism is obliterated by β-guanidinopropionic acid. Most striking in this respect is the observation that β-guanidinopropionic acid reduces the high rate of creatine uptake in isolated young red cells or metabolically activated normal red cell populations by a larger percentage than the low rate in older red cells or preparations of metabolically depleted red cell populations with normal age distribution. The absolute values of the transport rates in maximally inhibited cells are nearly the same, regardless of the age or metabolic state of the cells. The findings described suggest that the inhibitor only affects that portion of creatine transport which is dependent on cell metabolism. This portion is larger in younger than in older cells, while the other portion of creatine transport is independent of cell age and metabolism.

The two-pathway model of creatine transport explains the observation that the effects of metabolic depletion and inhibition by β-guanidinopropionic acid are pronounced at low creatine concentrations and may become negligibly small at high concentrations. At low concentrations, most of the transport takes place via the saturable pathway and hence is sensitive to metabolic depletion and the presence of the inhibitor. At high creatine concentrations, where the saturable transport system operates at its maximal rate while the other does not, the influence of the saturable system becomes too small to be detectable.

The need to discriminate between the two components of creatine transport is further demonstrated by the observations with the proteolytic enzyme pronase. At 0.5 mM creatine, this enzyme produces a maximal inhibition of approx. 40%. This inhibition is quite close to the maximal effect produced by β-guanidinopropionic acid and strongly suggests that the enzyme and the chemical modifier affect the same transport component. The fact that a proteolytic enzyme is effective points to the involvement of a transport protein in the mediation of the β-guanidinopropionic acid-sensitive component of creatine transport.

It is interesting to note that under suitable experimental conditions (p. 222) a range of proteolytic enzyme (trypsin, chymotrypsin, pronase at high concentrations) may produce an acceleration of creatine transport. The enhancement becomes most pronounced if the proteolytic enzymes are removed from the membrane by washing prior to the flux measurements. One simple explanation of this latter finding would consist of the assumption that the enzyme may stick to the partially digested transport protein and thus prevent the effect of the enzymatic digestion becoming apparent. The involvement of the active center of trypsin in the binding to the modified transport system is suggested by the observation that trypsin inhibitor prevents the inhibitory action of the enzyme.

The characteristics of creatinine transport are quite different to those of

creatine transport. The kinetics of penetration of creatinine are easily reconcilable with an equilibration across the membrane by passive transport in a two-compartment system: the time course of equilibration follows a single exponential, there is no indication of saturation kinetics, and neither β-guanidinopropionic acid nor proteolytic enzymes exert any effects. These findings confirm that the features of creatine transport described above are genuinely specific and that neither the chemical agent nor the enzymes produce generalized changes of the red cell membrane. Only phloretin was found to affect creatine and creatinine transport to about the same extent. However, this agent is well-known for the low specificity of its effects. It also inhibits, for example, the exchange of anions and the transport of sugars and simple physicochemical mechanisms have been proposed to explain its mode of action (for review, see Ref. 23).

The absence of an effect of hexanol on creatine transport may deserve a word of comment. This agent is known to accelerate the transport of solutes that penetrate preferentially via the lipid phase [23]. The absence of such acceleration suggests that creatine does not penetrate by dissolution in the lipid phase but by diffusion across pores or after combination with a low-affinity carrier.

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