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THE SEQUESTRATION OF Na^+, K^+ AND Cl^- IN THE CELLULAR NUCLEUS AND ITS ENERGETIC CONSEQUENCES FOR THE GRADIENT HYPOTHESIS OF AMINO ACID TRANSPORT IN EHRLICH CELLS*

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SUMMARY

In order to study the intracellular distribution of Na^+, K^+, Cl^- and 2-aminoisobutyrate, and to assess the true gradients of these solutes across the cellular membrane, Ehrlich cells were lyophilized and fractionated in nonaqueous solvents. It has been found that K^+ and 2-aminoisobutyrate are rather evenly distributed over the whole cellular space whereas the major part of Na^+ and Cl^- is closely associated with DNA, and hence appears to be appreciably sequestered in the nuclei. From the data it has been derived that the concentration of Na^+ in the cytoplasm must be up to 10 times lower than was previously assumed. Accordingly, the electrochemical potential difference of Na^+ can be expected to exceed previous estimates by at least 5000 J/mole and should therefore be approximately adequate to drive the accumulation of 2-aminoisobutyrate. With decreasing extracellular Na^+, the nuclear sequestration of this ion is diminished. It virtually disappears if the extracellular Na^+ is reduced to 30 mM or below, so that the cellular Na^+ need no longer be corrected for nuclear sequestration. This relation also holds if the gradient of K^+ and Na^+ is inverted by incubating cells preloaded with Na^+, in buffers with low Na^+ and high K^+. Since under such conditions α-aminoisobutyrate can still be transported actively into the cells, this transport must occur against the ion gradients. It is concluded that nuclear sequestration may account for the assumed deficit in osmotic driving forces under normal conditions, but does not explain how active 2-aminoisobutyrate accumulation may occur in spite of inverted alkali ion gradients.

INTRODUCTION

The active transport and accumulation of certain amino acids, notably of those belonging to the A system in Ehrlich ascites cells, depend on Na^+ [2–5]. The "gradient hypothesis", offered to explain such dependence postulates that the electro-

* Part of the results has been reported in preliminary form on "Herbsttagung (1972) der Gesellschaft für Biologische Chemie" in Erlangen-Nürnberg [1].

chemical potential gradient of Na^+, maintained by the Na^+ pump, is the immediate driving force for the amino acid transport [4, 6]. This energy transfer requires that the inflow of the amino acid is coupled to the inflow of Na^+ (co-transport), and possibly to the outflow of K^+ (counter-transport). In spite of ample evidence in favor of this kind of coupling it is still questioned whether these gradients cover the total driving force required, or whether some, or even the major, part is provided by direct (chemi-osmotic) coupling to metabolism [6, 7]. The following observations have been considered to support this latter view [7]:

(1) The electrochemical potential gradient of Na^+ has been assessed to be grossly inadequate to supply enough energy for the active accumulation of 2-aminoisobutyrate actually observed [8]. Even if the 2-aminoisobutyrate transport were as tightly coupled to the K^+ exit as it supposedly is to the Na^+ entry, the combined Na^+ and (inverse) K^+ gradients would barely be sufficient except on condition of an unrealistically high efficiency.

(2) Ehrlich cells accumulate glycine about 3 times as effectively during normal respiration than during metabolic inhibition, even with the same Na^+ (and K^+) gradients [5].

(3) Ehrlich cells may actively transport 2-aminoisobutyrate in the absence of or even with reversed alkali ion gradients [8, 9].

All these observations seem to reveal an additional driving force not accounted for by electrolyte gradients. The conclusion, however, that this extra force stems directly from a metabolic reaction, via chemiosmotic coupling, must be challenged on the following grounds. All ion gradients mentioned are based on the ion concentrations of the whole cell, and their validity hence rests on two tacit assumptions: (i) that the activity coefficient of Na^+ is approximately equal inside and outside the cell, (ii) that there are no intracellular compartments for Na^+, i.e. that Na^+ is evenly distributed over the whole cellular water.

As for the first assumption, evidence has been reported for other cells that the activity coefficients of alkali chlorides indeed differ very little inside and outside the cell. It has also been rigorously demonstrated that cellular proteins at concentrations occurring in the cell have an only slight effect on the activity coefficient of KCl, usually depressing it by about 10 %. This depression appears to be largely compensated for by non-solvent, i.e. protein-bound, water (ca. 0.3 ml/g protein) [10]. Also the activity coefficients of Na^+, if referred to total cell water, may safely be assumed to be so close to the corresponding ones in the medium that differences may be disregarded in the present context (Pfister, H., personal communication).

More doubtful is the second assumption, that of equal distribution of solutes in cellular space. If, for instance, part of cellular Na^+ were sequestered, be it by specific binding to colloids or undissolved structures, or by seclusion in compartments not in equilibrium with the cytoplasmic space, the true electrochemical potential gradient across the cellular membrane might considerably exceed the one previously derived from overall concentrations.

A previous attempt to explain apparent discordances with the gradient hypothesis on the basis of unequal distribution of Na^+ and K^+ in the cellular space has been made by Eddy et al. [4]. He assumed that a powerful Na^+–K^+ pump would produce a region depleted of Na^+ and enriched with K^+ adjacent to the inside face of the cell membrane. As a consequence the driving gradients of both Na^+ and K^+

across the cell membrane would be higher than estimated on the basis of the overall concentration of these ions within the cell. Since during metabolic inhibition of the pump this region could be expected to disappear, the observed metabolic effect on transport capacity of a given (overall) Na^+ and K^+ gradient could be easily accounted for. While such a hypothesis appears plausible at face value, it can be shown on the basis of realistic intracellular diffusion rates that such a region, should it occur, is only transient and must have disappeared long before it could be detected by the usual methods [11].

A more permanent compartmentalization may, however, be caused by an enrichment of Na^+, and possibly a depletion of K^+, within the cell nucleus. Preliminary experiments, in which cells were disrupted and fractionated in non-aqueous solvents, have shown that normally a substantial part of Na^+ is sequestered in the nucleus [11]. Consequently, the true cytoplasmic Na^+ concentration should be lower than has previously been assumed. The present experiments, designed to substantiate and extend such observations, have led to the conclusion that the true electrochemical potential gradient of Na^+, if corrected for nuclear sequestration, is adequate for the amino acid accumulation under normal, but not all, conditions.

MATERIALS AND METHODS

Incubation and fractionation of cells

The cells were disintegrated and fractionated with nonaqueous solvents by a method based on those previously developed for other cells by Behrens [12], Stern and Mirsky [13] and Siebert [14]. Since it turned out to be difficult to disrupt Ehrlich cells completely without damaging the nuclei, the original aim to recover intact nuclei was abandoned in order to secure exhaustive cell destruction. Solutes sequestered by nuclei will presumably stick to nuclear fragments after freeze-drying and treatment with non-aqueous fluids in which these solutes do not dissolve, as appears to be confirmed by the experimental results. After preincubation for 30 min in normal Krebs–Ringer phosphate (145 mM Na^+, 8 mM K^+, 1.9 mM Ca^{2+}, 1.3 mM Mg^{2+}, 138 mM Cl^-, 1.3 mM SO_4^{2-}, 10 mM P_i, 1 % albumin, adjusted to pH 7.4 with 0.31 M HCl) as previously described [9], the cells were incubated for 2 min with the labeled material in either normal Krebs–Ringer phosphate or in buffer solutions of reduced Na^+ concentration as indicated. The incubation was terminated by chilling to about 2 °C followed by centrifugation for 10 min at about $3000 \times g$ at 0 °C. The pellet was drained of attached extracellular fluid, frozen in liquid N_2 and lyophilized. The dry cellular mass, crushed and powdered in a Potter–Elvehjem homogenizer, was suspended in cold light petroleum and ground by shaking with glass beads for several hours until whole cells had virtually disappeared. The cell fragments were spun down and resuspended in mixtures of cyclohexane and carbon tetrachloride of defined densities. The fractionation was carried out in the first step by repeated centrifugations in mixtures of cyclohexane and carbon tetrachloride in the order of ascending density between 1.18 and 1.35 g/ml. In a second step, the heavier fractions were further resolved by centrifugation through a density gradient ranging between 1.31 and 1.56 g/ml. About 7–8 fractions of different density were usually obtained per sample.

Analysis of DNA, K^+, Na^+, Cl^-, and radioactivity

Each fraction was dryed, weighed and extracted with water overnight. The

protein was precipitated by cold trichloroacetic acid at a final concentration of 3 %; DNA was extracted from the residue according to the procedures of Schmidt and Thannhauser and of Schneider, as described by Leslie [15], and then assayed by the diphenylaminmethod of Dische [16] with an Eppendorf photometer. The DNA standard was kindly supplied by Professor R. K. Zahn, Mainz.

In the aqueous extracts the alkali ions were determined using an Eppendorf flame photometer, Cl^- with an Aminco-Cotlove Automatic Chloride Titrator, the radioactivity in a liquid scintillation counter (Packard).

Since DNA occurs almost exclusively in the nuclei it was used to monitor the amount of nuclear material in each fraction. The content of any solute accumulated in the nucleus should rise proportionally with the DNA content, and, from the proportionality factor or slope, the extra amount of solute accumulated per unit weight of nuclear material can be derived as is detailed in the Appendix. For this purpose the content of the solute, in μmoles/g dry wt, has been plotted versus the content of DNA in mg/g dry wt, for each fraction.

The sequestration per mg DNA has been taken to equal the slope of the curve obtained. This assumption is correct only if the water content per g dry wt is the same for cytoplasm and nucleus. The water:dry wt ratio of the nucleus is not precisely known for Ehrlich cells, but likely to be smaller than that of the cytoplasm. Hence, the slope represents only the lower limit of sequestration, as shown in the Appendix.

It should be mentioned that during the fractionation procedure with non-aqueous media lipids are removed, which may amount to about 10 % of the non-extracted dry material of all fractions [14]. The various contents of the fractionated material, if referred to the total dry weight of each fraction, should therefore be in reality somewhat lower than plotted in our charts. Since, however, this applies to the content of solutes and to that of DNA alike, and since, furthermore, the content of extractable lipids is supposedly similar in all fractions, the slopes should not, or not appreciably, be affected by the neglect of the lipid content.

RESULTS

Regular Krebs–Ringer phosphate buffer

After incubation in regular Krebs–Ringer phosphate buffer containing 140 mM Na^+, 15 mM K^+ and 1 mM 2-aminoisobutyrate for 2 min, the cells were separated, lyophilized, fractionated and analyzed as described above. Fig. 1 shows the contents of Na^+, K^+ and 2-[^{14}C]aminoisobutyrate plotted versus the DNA content of each fraction. It is seen that the levels of both K^+ and 2-aminoisobutyrate are similar in all fractions, independently of the DNA content. The slight decrease of 2-amino-isobutyrate with increasing DNA is presumably due to the fact that the water:dry weight ratio (w) of the nucleus is somewhat smaller than that of the cytoplasm. The Na^+ content of all fractions containing more than 50 mg/g DNA rises almost linearly with increasing DNA, presumably owing to sequestration of this ion in the nucleus. It is shown in the Appendix that the slope of this curve may be taken to represent the minimum amount of Na^+ sequestered per unit nuclear material. The fractions with a DNA content of 50 mg/g and less throughout have a considerably higher Na^+ content than could be extrapolated from the above mentioned slope.

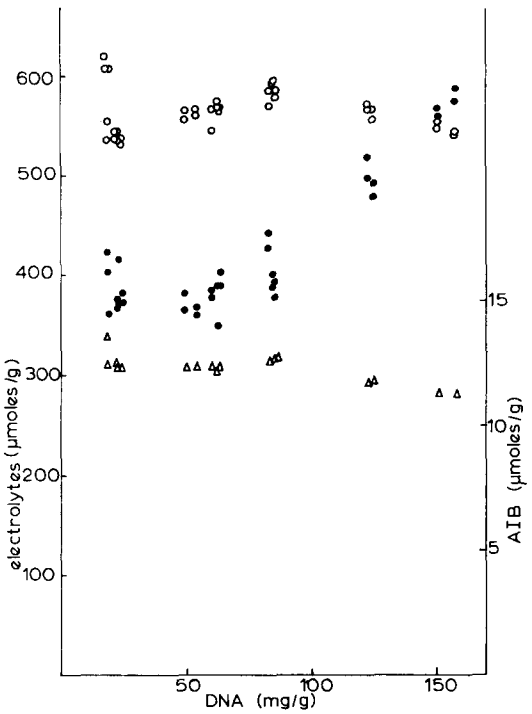

Fig. 1. Intracellular distribution of Na^+, K^+, and 2-aminoisobutyrate after incubation with high [Na^+] and low [K^+] buffer. Cells were incubated in a Krebs–Ringer phosphate buffer containing 138 mM Na^+ and 15 mM K^+ and 1 mM 2-aminoisobutyrate. The concentrations of the other components of the solution were identical with normal Krebs–Ringer phosphate buffer. The contents of Na^+ (●), K^+ (○), and 2-aminoisobutyrate (△) of each fraction are plotted versus the content of DNA (abscissa). Of each fraction two analyses each for K^+ and Na^+ have been carried out and plotted.

The extra content of these fractions can presumably be attributed to the high content of membrane material which after freeze-drying may be studded with the electrolytes of the trapped extracellular volume. From Fig. 1 it may be concluded that, under normal experimental conditions, both 2-aminoisobutyrate and K^+ are rather evenly distributed over the cellular water and that, consequently, their overall concentration is fairly close to the cytoplasmic concentration. In contrast, the cytoplasmic Na^+ concentration for the amount sequestered by the nucleus must be much lower than the corresponding overall concentration. The slope in this experiment is 2.0 μmoles Na^+ per mg DNA. The DNA content of the total cell being about 45 mg per g dry wt and the overall Na^+ content 109 μmoles/g dry wt, the corrected cytoplasmic Na^+ would be 19 μmoles/g dry wt, i.e. only 17 % of the overall cell Na^+.

Low Na^+ Krebs-Ringer phosphate buffer

Similar experiments were carried out with media in which K^+ was increased at the expense of Na^+. Fig. 2 shows a typical experiment, in which both [Na^+] and [K^+] were about 80 mM. The slope of Na^+ content versus DNA content is markedly reduced, whereas that of the corresponding K^+ curve rises slightly. The 2-amino-

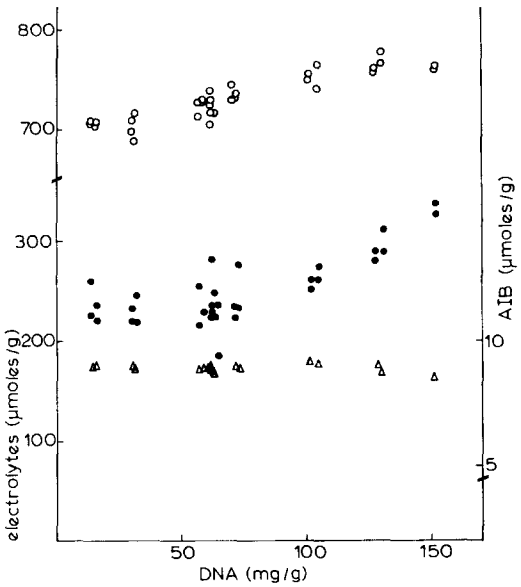

Fig. 2. Intracellular distribution of Na^+, K^+, and 2-aminoisobutyrate after incubation with buffer of intermediate $[Na^+]$ and $[K^+]$. Cells were incubated in modified Krebs–Ringer phosphate buffer containing 72 mM Na^+ and 80 mM K^+. The concentration of all other ions were identical with those in normal Krebs–Ringer phosphate buffer. The contents of Na^+ (●), K^+ (○), and 2-aminoisobutyrate (AIB) (△) of each fraction are plotted versus the content of DNA (abscissa). Of each fraction two analyses each for K^+ and Na^+ have been carried out and plotted.

isobutyrate content is not affected noticeably. If the $[Na^+]$ of the medium is reduced still further, to 41 mM, with a compensatory rise in $[K^+]$, the slope of the Na^+ content virtually disappears (Fig. 3) while the slope of the K^+ content becomes more prominent, amounting to about 0.9 μmoles/mg DNA. It looks as if K^+ is now sequestered whereas Na^+ appears to have an approximately equal concentration throughout the cellular water.

If choline is used instead of K^+ as a replacement for Na^+, the distribution of Na^+ over the cell behaves as described above. No rise in Na^+ is seen, whereas some sequestration of K^+ is observed (not shown).

Cl^- distribution

Cl^- also appears to be accumulated in the nucleus, as shown in Figs 3 and 4. The slopes for Cl^- appear to match the corresponding slopes of the cations that are sequestered. Hence, Cl^- must also be lower in the cytoplasm than has previously been assumed. The electrical potential difference across the cell membrane should accordingly be higher than estimated previously, but appears to decline with decreasing extracellular Na^+.

Inversion of Na^+ and K^+ gradients

In previous experiments, designed to study 2-aminoisobutyrate transport with inverted Na^+ and K^+ gradients, the conditions were similar to the present ones with low extracellular Na^+ and high extracellular K^+ [8, 9]. It should therefore be ex-

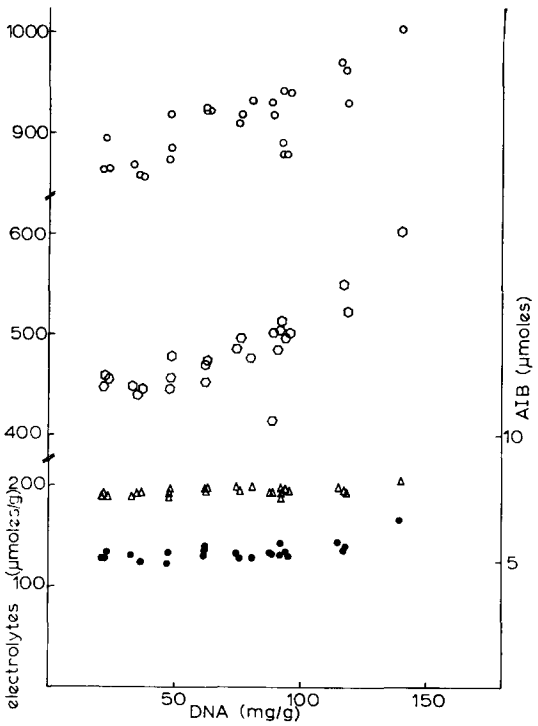

Fig. 3. Intracellular distribution of Na^+, K^+, Cl^- and 2-aminoisobutyrate after incubation with low [Na^+] and high [K^+] buffer. Cells were incubated in modified Krebs–Ringer phosphate buffer with 41 mM Na^+, 113 mM K^+, and 1 mM 2-aminoisobutyrate. The concentrations of the other ions are the same as in normal Krebs–Ringer phosphate. For each fraction, the contents of Na^+ (●), K^+ (○), Cl^- (◇), and 2-aminoisobutyrate (△) are plotted, versus the DNA content (abscissa).

pected that also in the latter the sequestration of Na^+ will vanish and that of K^+ will appear instead. In order to test whether this is true and, if so, whether 2-aminoisobutyrate is still actively transported, the experiments with inverted Na^+ and K^+ gradients have been repeated and extended to include determination of nuclear sequestration of these ions. Inversion of the Na^+ and K^+ gradients was achieved by treatment of the cells at 0 °C in half isotonic Krebs–Ringer phosphate and isotonic Krebs–Ringer phosphate and subsequent incubation in buffer solutions with low Na^+ and high K^+, as previously described [9]. In order to test for uphill 2-aminoisobutyrate transport, cells were loaded with the amino acid prior to the main incubation. Care had to be taken to keep the resulting opposed ion gradients within moderate ranges, i.e. below the critical value of about −4000 J/mole, at which the inward transport of 2-aminoisobutyrate was found to stop and to change into outward transport. After the main incubation the cells were lyophilized and fractionated as described above. The results are shown in Table I. It is seen that the opposing driving force, set up by the combined alkali ion gradients, is between −2000 and −4000 J/mole and that these values are not appreciably changed by correction for nuclear sequestration. However, 2-aminoisobutyrate was found to be transported actively, i.e. against its own chemical potential gradient.

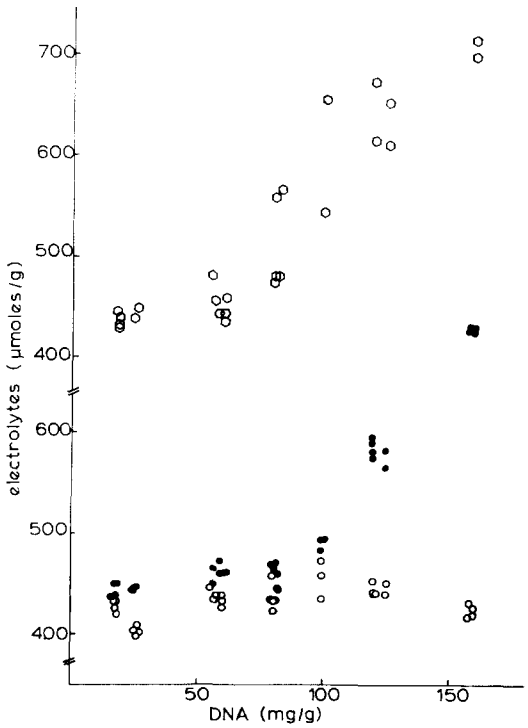

Fig. 4. Intracellular distribution of Na^+, K^+, and Cl^- after incubation with high [Na^+] and low [K^+] buffer. The experiment was carried out under similar conditions as that of Fig. 1, but with main emphasis on Cl^- distribution. Cells were incubated in normal Krebs–Ringer phosphate buffer containing 145 mM Na^+ and 8 mM K^+. The contents of Na^+ (●), K^+ (○), and Cl^- (◇) are plotted versus the content of DNA (abscissa). In each Fraction 2–4 analyses of each ion have been carried out and plotted.

Contamination with extracellular material

To investigate to what extent the various fractions are contaminated by extracellular solutes, the cells were suspended at 0 °C, prior to freeze-drying and fractionation, in a choline Krebs–Ringer phosphate buffer containing labeled P_i which had been found not to enter the cell appreciably under these conditions. Fig. 5 shows the pooled data of 2 experiments, the overall Na^+ and the extracellular Na^+ computed from the contamination by radioactive P_i found in the fractions. (Similar data were obtained with 2-aminoisobutyrate as a tracer of extracellular material.) Under these conditions the extracellular contamination is low and present at a similar level in all fractions, although somewhat elevated in the low DNA fractions which presumably contain the major part of the cell membrane. Obviously, enrichment of extracellular solutes in the nuclear material does not take place and can therefore not account for the apparent accumulation of Na^+ and Cl^- by the nucleus. A correction of the curves of Na^+, K^+, Cl^- and 2-aminoisobutyrate contents for extracellular contamination on the basis of the present results would shift the curves to lower concentration ranges, and the slopes of these curves might be slightly increased.

TABLE I

DISTRIBUTION OF THE ALKALI IONS BETWEEN MEDIUM AND CYTOPLASM AND THE ESTIMATED TRUE IONIC DRIVING FORCES FOR 2-AMINOISOBUTYRATE TRANSPORT

The cytoplasmic ion concentrations (Columns 5 and 6) were derived by the correcting of the corresponding overall concentrations (Columns 3 and 4) for the nuclear sequestration. The ionic driving forces (Columns 7 and 8) are estimated under the assumption that the amino acid transport is coupled to inward Na^+ flow and outward K^+ flow. Accordingly the negative driving force of K^+ is added to the positive one of Na^+. Under the assumption that the ratio between 2-aminoisobutyrate transport and each ion flux is 1:1 the electrical potential differences should cancel. Rows 1, 2, and 3 refer to the experiments presented in Figs 1, 2, and 3, respectively.

Concns (mM)						Ionic driving force ($X_{Na^+} - X_{K^+}$) (J/mole)	
Extracellular		Intracellular					
		Overall		Cytoplasmic			
Na^+	K^+	Na^+	K^+	Na^+	K^+	Uncorrected	Corrected
138	15	43	180	7.3	185	9400	14 050
72	80	33	193	16	186	4300	6 050
41	113	17	200	16	185	3740	3 690
31	132	83	149	77	127	−2230	−2 420
13	129	60	141	54	128	−3710	−3 690

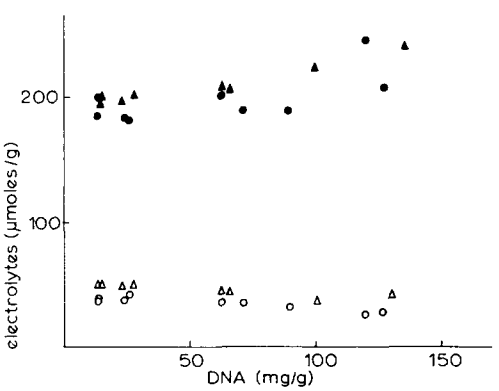

Fig. 5. Contamination of cellular fractions with extracellular material. Cells were incubated as before in normal Krebs–Ringer phosphate buffer. Before fractionation cells were briefly washed by suspension for 2 min in a Krebs–Ringer phosphate buffer solution in which all Na^+ had been replaced by choline and in which the phosphate was labeled by ^{32}P. The Na^+ content (solid symbols) is plotted versus the DNA content as before. The contribution of trapped extracellular Na^+ (open symbols), as derived from the content of ^{32}P in each fraction is also plotted versus DNA (abscissa). The triangles and circles refer to two independent experiments carried out under the same conditions.

DISCUSSION

Investigating the nuclear content of ions and amino acids, after fractionating the cells in non-aqueous media and relating the content of ions and amino acid to the DNA, we indeed found evidence that under normal conditions the nucleus contains much more Na^+ and Cl^- than does the cytoplasm. On the other hand, both K^+ and 2-aminoisobutyrate appear to be distributed rather equally all over the cell since their content is independent of the DNA content. It cannot be said yet whether the sequestration of Na^+ and Cl^- is due to specific adsorption or to an accumulation as free ions in secluded compartments. It is noteworthy in this context that the total osmolarity of Na^+, K^+ and Cl^- appears to be considerably greater in the nucleus than in the cytoplasm. This relation, together with the obvious preference of Na^+ to K^+, argues in favor of specific adsorption, since a major osmotic disequilibrium between nuclear and extranuclear fluid is unlikely. If Na^+ were specifically bound to negative DNA groups, the resulting excess of positive groups of the histones might account for a higher nuclear concentration of Cl^- owing to a Donnan distribution.

From the amount of Na^+ sequestered per mg DNA and from the total DNA content of the cell the freely dissolved Na^+ in the cell water was corrected and found to be lower than the overall Na^+ by a factor of 5–10 (Table I). The real correction is probably even lower as the sequestration of Na^+, derived from the slope of the Na^+ curve, is likely to be underestimated. This underestimate arises partly because the water:dry weight ratio of the nucleic material is probably higher than that of the cytoplasm (see Appendix), and partly because the contamination with extracellular Na^+ of the nuclear material seems lower than that of other fragments of the cell (Fig. 5).

The true driving force set up by the corrected Na^+ gradient between medium and cytoplasm would thus exceed the previously estimated value by at least 5000 J/mole under normal conditions. This value would easily cover any deficit in osmotic driving force so far suspected, provided that the amino acid distributes evenly over the major part of the total cellular space. If the latter were not true, if, for instance, the amino acid were accumulated in the cytoplasm only, the maximum accumulation ratio would be higher than estimated. In that case part or all of the increment in driving force would be compensated for by a parallel increment in the counter force set up by the 2-aminoisobutyrate gradient. The experimental results indeed appear to show an even distribution of 2-aminoisobutyrate all over the cell. To the extent that the observed sequestration of Cl^- in the nucleus means that the electrical potential difference across the plasma membrane is also higher than has previously been assumed, the electrochemical potential gradient of Na^+ alone, i.e. without the inverse K^+ gradient, might already suffice to supply all the driving force required for the amino acid accumulation.

The above corrections of the driving force set up by the Na^+ gradient apply only as long as the ionic composition of the incubation medium is normal. With decreasing extracellular Na^+ and a compensatory increase in K^+, the nuclear sequestration of Na^+ appears to decline; it disappears almost completely if the extracellular Na^+ is as low as 40 mM. Instead, the slope of the K^+ curve rises, as if K^+ now occupied the nuclear sites normally occupied by Na^+.

The response of the nuclear Na^+ to changes in extracellular Na^+ is rather fast, since washing of the cells for only a few minutes with Na^+-free solutions, subsequent to incubation in normal medium, and prior to freeze-drying, leads to a marked decrease in nuclear Na^+, at a time when the cytoplasmic ion concentration is hardly affected (Fig. 5). That nuclear electrolytes equilibrate with the extracellular rather than with the cytoplasmic fluid has been postulated for liver cells by Siebert et al. [17], with the implication that nuclear regions communicate directly with the extracellular space, possibly through channels of the endoplasmatic reticulum. Whether the same holds for Ehrlich cell nuclei cannot be said yet, though the observations with low extracellular Na^+ appear to be consistent with such a view.

Table I shows the tentative re-evaluation of the ion gradients taking into account the varying degree of Na^+ sequestration by the nucleus, and the corresponding driving forces, corrected accordingly. It is seen that at low extracellular Na^+ a correction of the Na^+ gradient appears no longer necessary, whereas that of K^+ would have to be corrected in the negative direction. In conclusion, we may briefly reconsider the main arguments against the gradient hypothesis, which are listed in the Introduction, in the light of the present findings: The first of these arguments, namely that the electrochemical potential gradients of Na^+ and K^+ are inadequate for the observed 2-aminoisobutyrate accumulation could be invalidated if nuclear sequestration of Na^+ and Cl^- are taken into account, as long as the ionic composition of the extracellular fluid is normal. Also the second argument, namely that in metabolically intact cells the same Na^+ gradients are more effective in transporting α-aminoisobutyrate than they are during metabolic inhibition may be refuted on the same grounds. It is plausible that during metabolic inhibition the cytoplasmic Na^+ could rise without much change in overall Na^+, in other words, that during and without metabolic inhibition seemingly equal Na^+ gradients are not really equal. The third of the mentioned arguments, based on the observation that 2-aminoisobutyrate can be actively transported into the cells while both Na^+ and K^+ gradients are inverted, is perhaps the strongest one. One might speculate that in these cases, owing to sequestration of Na^+in, and possibly exclusion of K^+ from, the nucleus, the gradients of the alkali ions between medium and cytoplasm merely appeared to be inverted but were still in the normal direction. On the other hand, at the low extracellular Na^+ concentrations required to invert the Na^+ gradient, nuclear sequestration of this ion can hardly be expected from what has been said. Special experiments have therefore been carried out in order to check whether, under the conditions studied by Schafer and Heinz [9], the alkali ion gradients are really inverted and whether active uptake of 2-aminoisobutyrate really occurs. The results confirmed both: nuclear sequestration of Na^+ gradient could not be detected, hence the inversion of the Na^+ gradient should have been real. The moderate nuclear sequestration of K^+ that was observed instead indicated that the inversion of the K^+ gradient was even greater than anticipated. Under the same conditions, the amino acid could clearly be shown to be taken up actively, i.e. against its own concentration gradient. Hence the argument raised by Schafer and Heinz against the gradient hypothesis, namely that 2-aminoisobutyrate may be transported actively in spite of (moderately) opposing ion gradients cannot be invalidated on the basis of nuclear sequestration. The effect of the observed sequestration is visualized in Fig. 6, in which net 2-aminoisobutyrate transport is plotted versus the raw and corrected osmotic driving forces derived from the

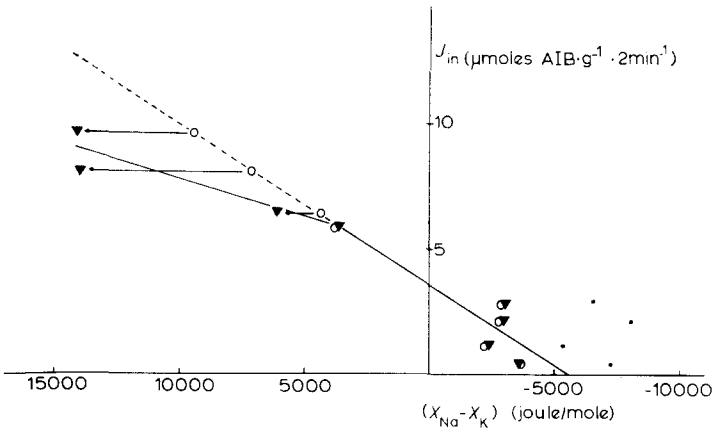

Fig. 6. The net influx of 2-aminoisobutyrate as a function of the ionic driving forces. The final results of 8 typical experiments with varying Na^+ and K^+ gradients are summarized. Abscissa: $X_{Na^+} - X_{K^+}$ the sum of the ionic driving forces of Na^+ and K^+, in J/mole. Ordinate: Influx of 2-aminoisobutyrate (J_{AIB}) in μmoles/g per 2 min. ○, uncorrected driving forces; ▲, corresponding driving forces corrected for nuclear sequestration of the alkali ion. The corresponding values are connected by a horizontal arrow. Since in the experiments with inverted gradients (right side of the ordinate) the intracellular concentrations of Na^+ and K^+ changed considerably towards normalization of the gradients during the 2-min incubation, the initial values are also given (●). Hence the intermediate values are somewhere in between, in any case more towards the right from the final (corrected) values. Each pair of values represents a whole experiment.

present experiments in a similar fashion as was done by Schafer and Heinz [9]. If the amino acid transport were driven exclusively by the ion gradients, the regression line should pass through the origin. It is seen, however, that in spite of substantial corrections in the range of the normal gradients (left side), the regression line shows no tendency towards the origin, and in the range of inverted gradients its course hardly differs from that drawn by Schafer and Heinz. The line seems to cut the abscissa at about −5000 J/mole; in other words, the 2-aminoisobutyrate transport is stopped or reversed by an osmotic counter force of that magnitude. The question of where the extra energy required to overcome this counter-force originates is still open although a direct coupling of 2-aminoisobutyrate transport to ATP hydrolysis can probably be excluded [18].

APPENDIX

Estimation of sequstration of a solute (A) in the nucleus.

Symbols:

c = concentration, per unit H_2O volume;
u = concentration, per unit dry weight;
v = volume of H_2O space;
m = dry mass of solid;
the superscripts $'$ and $''$ refer to cytoplasm, and nucleus, respectively;
the superscript t to the total cell;
the subscript a refers to solute A, the subscript d to DNA;

w', w'', w^t = water/dry wt (dw) ratio of cytoplasm, nucleus and total cell, respectively; c_a'' the nuclear A concentration is treated here as consisting of two parts, one identical with the cytoplasmic A concentration, c_a', and the other, $\Delta c_a''$, representing the extra A of the nucleus, so that

$$c_a'' = c_a' + \Delta c_a'' \tag{1}$$

It is irrelevant in this context whether $\Delta c_a''$ stands for a real concentration difference, i.e. of freely dissolved solute, or for the amount of A attached to nuclear solids relative to the nuclear water.

$$\Delta c_a'' = \Delta u_a'' \cdot \frac{m''}{v''} \tag{2}$$

It is assumed that DNA occurs in nucleus only, and that the content of DNA per unit dry weight of nucleic material (u_d'') is the same in all fractions; in case of sequestration the content of extra solute should be proportional to the nuclear DNA content in each fraction of the cell

$$\Delta u_a'' = \beta u_d'', \tag{3}$$

β being the proportionality coefficient.

Combining Eqns 1, 2 and 3, we obtain

$$u_a'' = c_a' \cdot \frac{v''}{m''} + \Delta u_a''$$

or

$$u_a'' = \frac{m'}{m''} \frac{v''}{v'} u_a' + \beta u_d'' \tag{4}$$

It follows for the amount of A per total cellular dry weight

$$u_a^t = \frac{u_a' m' + u_a'' m''}{m^t}$$

or

$$u_a^t = \frac{u_a' m' \left(1 + \frac{v''}{v'}\right) + \beta u_d'' m''}{m^t} \tag{5}$$

Replacing u_a' by $c_a' \cdot w'$ in Eqn 4 and solving for c_a' we obtain

$$c_a' = \frac{u_a^t - \beta u_d^t}{w^t} \tag{6}$$

In our experiments β has for simplicity been taken to be equal to the slope. Strictly spoken, this assumption would hold only if the water dry weight ratio (w') of the cytoplasm were equal to that of the nucleus (w''), i.e. if

$$w' = w'' = w^t$$

Since, however, in all probability the nucleus is more compact than the cytoplasm, so that

$$w' > w^t > w''$$

β will almost certainly exceed the slope (s). It can be evaluated accordingly if Eqn 5 is applied to each fraction and differentiated for the DNA content. The slope(s) of the function obtained would then be

$$s = \frac{du_a^t}{du_d^t} = -\frac{u_a'}{u_d''}\left(1 - \frac{w''}{w'}\right) + \beta \tag{7}$$

Replacing again u_a' by $c_a' w'$ and dissolving for β we obtain

$$\beta = s + \frac{c_a'}{u_c''}(w' - w'') \tag{8}$$

which can be inserted into Eqn 6 to give the true cytoplasmic concentration:

$$c_a' = \frac{u_a^t - s \cdot u_d^t}{w'} \tag{9}$$

Since w' is not exactly known but will in all probability exceed w^t, equating with the slope(s) in Eqn 5 will give the minimum sequestration and, accordingly, the maximum value of c_a' only.

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