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The Maintenance of Adenine Nucleotide Levels During Kidney Storage in Intracellular Solutions (40469)

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In preserving organs for transplantation, cellular survival during storage is extremely important. Evidence is accumulating that preservation of the adenine nucleotide pool is a good indicator of organ viability (1, 2). However, the evidence is inconclusive in kidney storage because of lack of information on the normal adenine nucleotide level in kidney. It is thought that in the normal cell, the adenine nucleotide energy charge (3) should be between 0.8 to 0.95 (4). All values we have seen in the literature are lower than this (probably indicating some ATP breakdown during isolation). When these normal values are unreliable, it is difficult to make statements about the experimental values.

It has recently been shown that organ storage in "intracellular solutions" (solutions which have ionic concentrations that mimic the intracellular constitution of the cell) of Collin's and Sacks' allows the cell to maintain cell function during hypothermic storage for longer times than storage in extracellular solutions such as saline or Ringers lactate (5–7). The reason these intracellular solutions extend storage times remains obscure. Several possibilities have been suggested, including: (a) Maintaining cellular ATP levels by reducing the energy require to maintain membrane ionic concentration gradients; (b) reducing cellular edema; (c) preservation of intracellular potassium.

In this paper, we would like to present what we feel are better normal values for the concentrations of adenine nucleotides in kidneys and to examine the effect of various storage solutions on the adenine nucleotide levels of stored kidneys. Also, we would like to present evidence for an additional possible preservational function of intracellular solutions; which is the maintenance of the total adenine nucleotide pool by reducing the breakdown of adenosine monophosphate.

Methods. Mice (Charles River Laboratories) were used as the experimental animals; they were anesthetized with 70 μg/g sodium pentobarbitol. The mice were opened with a midline incision and the kidneys snap-frozen by compressing a portion of the kidney with pliers precooled in liquid nitrogen. The frozen section of kidney was quickly weighed and ground in a mortar and pestle which had been precooled to −70°. The powdered kidney was then homogenized in ice-cold 0.4 *M* percholoric acid. The acid insoluble material was removed by centrifugation (1800*g* for 15 min) and the extract carefully neutralized with KOH. The insoluble potassium perchlorate was removed by centrifugation (1800*g* for 15 min).

For stored kidneys, the mice were anesthetized and opened as before and the kidneys rapidly flushed with cold (0–4°) storage solution. Flushing the kidney was accomplished by cutting the renal vein and injecting 10 ml of cold storage solution into the left ventricle. The flush is judged to be adequate if there is significant paling of the kidney. The entire procedure takes 15–30 sec. The flushed kidney was snap-frozen and the nucleotides extracted as above. The other kidney was placed in the storage solution and maintained at 0–4° for either 2 or 48 hr. The stored kidneys were then snap-frozen and the nucleotides extracted as above. We have found that after flushing or storing the kidneys, it makes very little difference if the kidneys are snap-frozen or just quickly homogenized in cold perchloric acid; both methods were used in this paper. However, to get good normal values, snap-freezing and speed in processing are essential.

ATP was measured in these extracts using the firefly assay of Stanley and Williams (8). The firefly lanterns were purchased from Sigma Chemical Co., St. Louis, MO. Each vial was reconstituted with 5 ml of water and insoluble material was removed by centrifug-

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ing at 10,000*g* for 10 min. The reaction mixture contained, in a total volume of 3.15 ml: 0.10 mmoles sodium arsenate, pH 7.4; 0.044 mmoles $MgSO_4$; 0.01 mmoles potassium phosphate, pH 7.4; ATP, 10^{-9} to 10^{-12} moles, or tissue extract; and 0.15 ml firefly extract. The reaction was initiated by adding the firefly extract. One minute after initiation of the reaction, the amount of light released during a six second interval was measured.

ADP and AMP were measured using the linked enzymatic assay of Adams (9). ADP was measured by linking the pyruvate kinase reaction to the oxidation of DPNH by lactic dehydrogenase and measuring the decrease in UV absorbance as DPNH is converted to DPN. AMP was measured by linking both of these reactions to the myokinase reaction which converts AMP plus ATP to ADP. The energy charge, which defines the energy state of the cell in terms of the ratio of ATP to AMP was calculated according to Atkinson (3) for each storage condition.

Intracellular storage solutions tested were Collins' solution C_2 (5) and Sacks' solution (6). Collins solution does not contain heparin, phenoxybenzamine or procaine (as his earlier solution did) but does contain high potassium (115 m*M*) and sucrose (126 m*M*) concentrations. The osmolarity of Collins' solution is 320 mOsm/L. Sacks' solution is a hyperosmolar solution containing no sucrose but high potassium (126 m*M*) and mannitol (208 m*M*) concentrations. The osmolarity of Sacks' solution is 430 mOsm/L. Extracellular solutions used were saline (0.9% w/v NaCl in distilled water) and Ringer's lactate (27).

Enzymes (pyruvate kinase, lactic dehydrogenase, and myokinase) and nucleotides (ATP, ADP and AMP) were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals are reagent grade.

Results. Table I presents what we feel are more realistic values for the adenine nucleotide levels in normal kidneys. The energy charge value of 0.84 is within the generally accepted range for normal tissues (4), and considerably higher than values previously reported for kidney (10–12). Table I also shows the large decrease in the energy charge which occurs with flushing the kidney with a storage solution. In our hands flushing the kidney takes between 15–30 sec and results in a 45–50% decrease in the amount of ATP in the cell with an increase in ADP and AMP. There is very little change in the total adenine nucleotide level and therefore, a significant drop in the energy charge. These changes occur regardless of the composition of the flushing solution.

Table II demonstrates that these trends in the adenine nucleotide levels seen after flush-

TABLE I. ADENINE NUCLEOTIDE LEVELS IN MOUSE KIDNEYS AFTER FLUSHING WITH STORAGE SOLUTIONS.[a]

Flushing solution	ATP (mmoles/g)	ADP (mmoles/g)	AMP (mmoles/g)	TAN[c] (mmoles/g)	E.C.[c]
None[b]	1.896	0.625	0.114	2.635	0.84
Saline	1.014	0.720	0.575	2.309	0.60
Ringer's lactate	1.061	1.063	0.709	2.822	0.56
Sacks'	1.030	0.968	0.892	2.651	0.53
Collin's	0.946	0.858	0.794	2.597	0.53

[a] Each value is the average of at least 8 different kidney preparations.
[b] None under flushing solution is the normal values.
[c] TAN = total adenine nucleotides = (ATP) = (ADP) + (AMP); E.C. = Energy Charge.

TABLE II. ADENINE NUCLEOTIDE LEVELS IN MOUSE KIDNEY AFTER TWO HOURS OF COLD STORAGE.[a]

Storage solution	ATP (mmoles/g)	ADP (mmoles/g)	AMP (mmoles/g)	TAN[c] (mmoles/g)	E.C.[c]
Saline	0.417 (22%)[b]	0.543 (87%)	1.598 (1402%)	2.558 (97%)	0.27
Ringer's lactate	0.471 (25%)	0.681 (109%)	1.233 (1402%)	2.385 (91%)	0.34
Collin's	0.655 (35%)	0.495 (79%)	1.113 (976%)	2.262 (86%)	0.40
Sack's	0.533 (28%)	0.715 (114%)	1.524 (133%)	2.772 (105%)	0.32

[a] Kidneys were flushed with the cold storage solution and stored for 2 hr cold (0–4°). Each value is the average of at least 3 different kidney preparations.
[b] The values in parenthesis are the percent of the normal values.
[c] TAN = total adenine nucleotides = (ATP) = (ADP) + (AMP); E.C. = Energy charge.

ing kidneys continue during the first 2 hr of storage. The ATP level continues to fall, although at a slower rate and the ADP level fall back down to normal values as the high energy supply of the cell is exhausted. The AMP level continues to rise (up to 10 times normal level) while the total adenine nucleotide level remains near normal levels. The energy charge drops to less than half of the normal value. These changes occur in all storage solutions; the intracellular solutions give no increased protection over saline or Ringer's lactate for 2 hr of cold storage.

Table III confirms earlier reports (1, 10, 11, 13, 14) that storage in the intracellular storage solution of Collins' improves the total adenine nucleotide content of the cell when compared to storage in saline or Ringer's lactate. Table III also shows that similar results are given by storage in the intracellular solution of Sacks'. Kidneys stored in these solutions for 48 hr have almost twice the level of adenine nucleotides as kidneys stored in saline. Forty-eight hours of storage was chosen as the maximum storage time because this appears to be the maximum length of time kidneys can be stored successfully in Collins' solution while storage for this period of time in saline or Ringer's lactate is fatal to the kidney (5, 15). The important point is that the increase in cellular adenine nucleotide levels after storage in intracellular solutions is due to the increased amount of AMP retained by the cell, as there is very little improvement in the ATP or ADP content. AMP levels after 48 hr of storage in intracellular solutions are still 4.7- to 6.9-fold higher than normal kidneys while they are only 1.8- to 2.2-fold higher after storage in extracellular solutions.

It has been suggested that administration of purine nucleotide precursors or catabolic inhibitors may improve organ storage (10, 16, 17). However, adding the adenine nucleotide precursor inosine to the saline storage solution did not improve the total adenine nucleotide content of the cell during storage (Table IV). The values obtained for saline plus 10 m*M* inosine for all nucleotides corresponds very closely at all time points to values obtained for saline alone. Addition of allopurinol (a compound which inhibits the breakdown of purine nucleotides) at 500 μg/ml to the saline storage solution also had no effect on the adenine nucleotide content of the cell during storage (Table IV). Values obtained with saline plus allopurinol are no different than with saline alone.

Discussion. The results presented in this paper support the theory that one of the

TABLE III. ADENINE NUCLEOTIDE LEVELS IN MOUSE KIDNEYS AFTER FORTY-EIGHT HOURS OF COLD STORAGE.[a]

Storage solution	ATP (mmoles/g)	ADP (mmoles/g)	AMP (mmoles/g)	TAN[c] (mmoles/g)	E.C.[c]
Saline	0.139 (7%)[b]	0.139 (22%)	0.210 (184%)	0.488 (19%)	0.43
Ringer's lactate	0.061 (3%)	0.205 (33%)	0.252 (221%)	1.518 (20%)	0.16
Sack's	0.149 (8%)	0.280 (45%)	0.534 (468%)	0.973 (37%)	0.30
Collin's	0.116 (6%)	0.211 (34%)	0.787 (690%)	1.114 (42%)	0.20

[a] Kidneys were flushed with the cold storage solution and stored for 48 hr cold (0–4°). Each value is the average of at least 3 different kidney preparations.

[b] The values in parenthesis are the percent of the normal values.

[c] TAN = total adenine nucleotides = (ATP) = (ADP) + (AMP); E.C. = Energy charge.

TABLE IV. ADENINE NUCLEOTIDE LEVELS IN MOUSE KIDNEYS AFTER COLD STORAGE IN SALINE PLUS ADDITIVES.[a]

Storage solution	Time of Storage	ATP (mmoles/g)	ADP (mmoles/g)	AMP (mmoles/g)	TAN[c] (mmoles/g)	E.C.[c]
Saline + 10 m*M*	2 hr	0.418 (27%)[b]	0.600 (96%)	1.117 (980%)	2.235 (85%)	0.37
Inosine	48 hr	0.062 (3%)	0.169 (27%)	0.245 (215%)	0.476 (18%)	0.31
Saline + 500 μg/ml	2 hr	0.432 (23%)	0.518 (83%)	1.206 (1058%)	2.156 (82%)	0.32
Allopurinol	48 hr	0.119 (6%)	0.233 (37%)	0.332 (292%)	0.684 (26%)	0.34

[a] Kidneys were flushed with cold storage solution and stored for the indicated times at (0–4°). Each value is the average of at least 3 different kidney preparations.

[b] The values in parenthesis are the percent of normal values.

[c] TAN = total adenine nucleotides = (ATP) + (ADP) + (AMP); E.C. = Energy charges.

advantages of intracellular storage solutions, such as those described by Collins' and Sacks' is their ability to maintain adenine nucleotide levels in the cell during storage. The mechanism by which they do this is not entirely clear. It has been suggested that since these solutions approximate the intracellular cation concentrations, the amount of energy (ATP) the cell must use to maintain the proper internal ionic composition is reduced (5). However, although the total adenine nucleotide levels remain higher after storage in intracellular solutions, there is no difference in the ATP levels of kidneys stored for 48 hr in either saline (or Ringer's lactate) or in Collins' or Sacks' solutions, which would tend to rule out this suggestion. The total adenine nucleotide levels, however, are higher after storage for long periods in Collins' or Sacks' solutions. This is due entirely to higher AMP levels, which would suggest an alternate mechanism for the improved storage characteristics of the intracellular solutions; one in which the breakdown of purine nucleotides is reduced. Since adenine nucleotides are catabolized by the deamination or dephosphorylation of AMP, an increased level of AMP would indicate the intracellular storage solutions decrease the rate of purine nucleotide catabolism. Maintaining intracellular AMP levels would be a big advantage to the organ after reimplantation. The AMP would be available immediately for rephosphorylation in the mitochondria and the ATP levels could, therefore, be rapidly restored.

In this regard, it is interesting to note that adding a nucleotide precursor to saline did not improve the adenine nucleotide levels during storage. This would indicate that in order for these compounds to be effective, they may have to be given either prior to removal of the organ for storage (17) or after reimplantation (16) and not just added to the storage solution. Allopurinol in the storage solution also does not improve the adenine nucleotide levels. This is not surprising as there is no xanthine oxidase in kidneys (18), and would suggest that for this compound to be effective in renal transplantation, it must be preinjected into the animal (10, 19) where it can inhibit liver xanthine oxidase and build up the nucleotide pool prior to removal of the kidney.

It is also noteworthy that storage of kidneys in Collins' or Sacks' solution does not give any improvement in the energy charge values over storage in saline or Ringer's lactate. It has been postulated that the energy charge is a major regulatory factor in cell metabolism (3) and an indicator of cell viability during hemmorrhagic shock (19). However, it is well documented that storage in Collins' solution preserves kidney function better than in Ringer's lactate (20–23) and since we find no difference in the energy charge between the two groups, it is unlikely that energy charge can be used as an indication of cell survival during storage.

We submit, as a working hypothesis, the following sequence in adenine nucleotide metabolism during hypothermic organ storage. When the oxygen supply is cut off, oxidative phosphorylation in the mitochondria ceases and ATP production is stopped. The ATP level falls as it is used in the energy-requiring reactions of the cell. As the ATP level falls, *de novo* purine biosynthesis, an ATP requiring process, slows down and since catabolism continues unrestricted, the total adenine nucleotide level of the cell declines. The cell can withstand depressed levels of ATP for a period of time (24, 25) and it appears the length of time a cell is exposed to low ATP levels is more important than the absolute level of the ATP. Maintaining the adenine nucleotide level is important because the cell must have AMP available for phosphorylation when the oxygen supply is restored. If the total adenine nucleotide level has fallen below some critical level, ATP levels cannot be restored by the mitochondria due to the absence of AMP as a substrate for oxidative phosphorylation. *De novo* purine synthesis, therefore, remains depressed due to lack of energy source. Cellular integrity, which is dependent upon ATP, is disrupted and cell death eventually occurs. Storing the kidneys in Collins' or Sacks' maintains the total adenine nucleotide concentrations at higher levels for longer times than storage in saline or Ringer's lactate, thus allowing the cell to restore the ATP level and recover biochemical function upon reintroduction of oxygen.

Summary. The total adenine nucleotide content of mouse kidneys was higher when the organs were preserved by hypothermic

storage in the intracellular solutions of Collins' and Sacks' than when the kidneys were stored in saline or Ringer's lactate. This increase was due to an increased level of AMP in the cell. It is thus suggested that intracellular solutions maintain adenine nucleotide concentrations by preventing catabolism of AMP, thus keeping more AMP available for rephosphorylation after restoration of the blood supply. Adding inosine or allopurinol to saline did not increase the level of adenine nucleotides in stored kidneys.

Note added in proof. While this manuscript was in preparation Skjoldal and Bakke published an article (26) suggesting that energy charge was of little significance in predicting survival of *Cirolana borealis* during anoxia, which supports our results in this paper that total adenine nucleotide levels are more important in cell survival of ischemia than ATP levels or energy charge.

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