Evidence for enhanced uptake of ATP by liver and kidney in hemorrhagic shock

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CHAUDRY, IRSHAD H., MOHAMMED M. SAYEED, AND ARTHUR E. BAUE. *Evidence for enhanced uptake of ATP by liver and kidney in hemorrhagic shock.* Am. J. Physiol. 233(3): R83–R88 or Am. J. Physiol.: Regulatory Integrative Comp. Physiol. 2(2): R83–R88, 1977. – It has been shown that infusion of ATP-$MgCl_2$ proved beneficial in the treatment of shock; however, it is not known whether this effect is due to improvement in the microcirculation or direct provision of energy or a combination of the above or other effects. To elucidate the mechanism of the salutary effect of ATP-$MgCl_2$, we have now examined the in vitro uptake of ATP by liver and kidney of animals in shock. Rats were bled to a mean arterial pressure of 40 Torr and so maintained for 2 h. After the rats were killed, liver and kidney were removed and slices of tissue (0.3–0.5 mm thick) were incubated for 1 h in 1.0 ml of Krebs-HCO_3 buffer containing 10 mM glucose, 5 mM $MgCl_2$, and 5 mM [8-^{14}C]ATP or 5 mM [8-^{14}C]ADP, or 5 mM [8-^{14}C]AMP, or 5 mM [8-^{14}C]adenosine in 95% O_2-5% CO_2 and then homogenized. Tissue and medium samples were subjected to electrophoresis to separate and measure the various nucleotides. The uptake of [^{14}C]ATP but not that of [^{14}C]ADP or [^{14}C]adenosine by liver and kidney slices from animals in shock was 2.5 times greater than the corresponding uptake by control slices. Thus, the beneficial effect of ATP-$MgCl_2$ in shock could be due to provision of energy directly to tissue in which ATP levels were lowered.

ATP uptake; ADP uptake; adenosine; inosine

PREVIOUS WORK from our laboratory (2) and by other investigators (7, 12–15, 18) has shown a significant reduction in the ATP levels in liver and kidney of animals subjected to severe shock. Subsequently we found that this reduction in ATP levels is a progressive change during shock with not only a decrease in ATP, but with dephosphorylation of ADP, AMP, and creatine phosphate as well. The studies of Talaat et al. (23) and of Sharma and Eiseman (21) have shown that ATP infusion was beneficial to rats in hemorrhagic shock if given prior to bleeding, but not if administered thereafter. Our studies extended these observations and showed that administration of ATP-$MgCl_2$ before, during, or after a prolonged period of severe shock had a beneficial effect on the survival of rats (3). This did not occur when ADP-, AMP-, or adenosine-$MgCl_2$ was given (3). Although we have demonstrated a salutary effect of ATP-$MgCl_2$ administered in shock, we do not know whether this is due to improvement in the microcirculation, direct provision of energy to tissues in which ATP levels were lowered, combination of these factors, or other effects.

It has been shown that [^{14}C]ATP can enter intact skeletal muscle cells (1) and we have recently shown that this high-energy phosphate compound can also enter liver and kidney slices from normal animals (6). In the present study, we have examined [^{14}C]ATP uptake by tissues of animals subjected to hemorrhagic shock. In addition to the uptake of [^{14}C]ATP, we have investigated [^{14}C]ADP, [^{14}C]AMP, and [^{14}C]adenosine uptake by liver and kidney. The results to be presented indicate that ATP uptake by liver and kidney slices from animals in shock was at least 2.5 times greater than the corresponding uptake by control slices. Thus, the beneficial effect of ATP-$MgCl_2$ to animals in shock could be through direct provision of energy to cells in which ATP levels were lowered.

METHODS

Hemorrhagic shock procedure. Albino Holtzman rats, weighing 220–225 g, were fasted for 16 h prior to the experiment but were allowed water ad libitum. The rats were anesthetized lightly with ether and both femoral arteries were cannulated with polyethylene tubing (PE-50). Heparin, 400 units, was injected into each femoral artery. One cannula was connected to 1.75 m length of PE-50 tubing, which was calibrated in Torr, for monitoring mean arterial blood pressure. The other cannula was connected to a 10-ml heparinized glass syringe that served as a blood reservoir. The animals were restrained in the supine position and, when awake, were bled rapidly within 10 min to a mean arterial pressure of 40 Torr. This pressure was then maintained for 2 h, which required returning about 60% of the shed blood. The above method of measuring blood pressure was compared to another method using a Hewlett-Packard pressure transducer, model 267BC, and a Sanborn 150 recorder, and blood pressure was found to be identical both under control conditions (i.e., before bleeding) and at various stages of hemorrhage. At the end of 2 h, the animals were killed and their livers and kidneys removed. Tissues from these animals will be designated "shock" tissues. Tissues designated as controls were obtained from fasted rats which were anesthetized, cannulated, heparinized, and allowed to awaken but were not bled (6).

Incubation procedures. Liver and kidney slices were prepared and incubated as described in a recent paper (6). The organs were divided into small blocks (about 5 mm^3). They were then transfered to oxygenated (95% O_2-5% CO_2) Krebs-Henseleit bicarbonate buffer (pH 7.4). Slices from the liver and kidney were then prepared from the tissue blocks with razor blades (0.3–0.5 mm thick, avg wt 35 mg) within 5–8 min after excision. Two to four slices from each organ were incubated for 1 h at 37°C in 1.0 ml of Krebs-Henseleit bicarbonate buffer (pH 7.4) containing NaCl (118 mM), KCl (4.8 mM), $CaCl_2$ (2.6 mM), $MgSO_4$ (1.2 mM), KH_2PO_4 (1.2 mM), $NaHCO_3$ (25 mM), 10 mM glucose, and one of the following: 5 mM [8-^{14}C]ATP (0.45 μCi/μmol), 5 mM [8-^{14}C]ADP (0.25 μCi/μmol), 5 mM [8-^{14}C]AMP (0.25 μCi/μmol), or 5 mM [8-^{14}C]adenosine (2 μCi/μmol) under an atmosphere of 95% O_2-5% CO_2 in each case. The same buffer was used for control liver and kidney slices (6). Since ATP is a complexing agent (16, 19) and when added alone may chelate divalent cations from the tissues, an equimolar amount of $MgCl_2$ was added together with the nucleotide. Following incubation, slices were removed, rinsed quickly in ice-cold water, blotted on a dampened filter paper, and frozen between aluminum blocks chilled in dry ice. The slices were then homogenized in 1.0 ml of a solution containing trichloroacetic acid (10%)-HCl (0.1 M) and centrifuged.

Electrophoretic separation and calculations of nucleotide concentration. The supernatant solution was extracted 4 times with water-saturated ether and then neutralized with 1.0 M tris(hydroxymethyl)aminomethane (Tris) base. Samples (50 μl) of tissue extract and incubation medium were separately applied to a Whatmann 3MM paper and overspotted with 10 μl of a marker solution containing 0.05 μmol each of ATP, ADP, AMP, adenosine, IMP, inosine, adenine, and hypoxanthine. Following electrophoretic separation using the system described by Wadkins and Lehninger (24), the individual nucleotide[1] spots were detected under ultraviolet light, cut from paper, and placed in a counting vial together with 15 ml of an aqueous scintillation solution. Radioactivity was counted in a Packard liquid scintillation counter. Approximately 90–95% of the radioactivity applied to the electrophoretogram was recovered.

The concentration of adenine and hypoxanthine nucleotides in medium (μmol/ml) and slices (μmol/g) were calculated from the radioactivity observed in each fraction. A nucleotide was considered to have an intracellular distribution when the total tissue content exceeded the extracellular content. Extracellular concentrations were calculated on the assumption that the concentration in the extracellular water was the same as that of the medium.

Extracellular space was determined in separate experiments as follows: liver and kidney slices were incubated for 1 h at 37°C in 1.0 ml of Krebs-Henseleit buffer (pH 7.4) containing 2.5 mg/ml inulin. Following incubation slices were rinsed in ice-cold water, blotted, and frozen between aluminum blocks cooled in dry ice. A protein-free extract was prepared by homogenizing the tissue in $Ba(OH)_2$-$ZnSO_4$. Inulin was measured by the method of Roe et al. (17).

[1] To avoid repeated qualification nucleotide will also include adenosine and inosine where applicable.

RESULTS

Extracellular spaces of shock liver and kidney slices. The extracellular spaces of liver and kidney slices from animals in shock measured after 1 h incubation of 37°C using inulin as a marker were found to be 0.30 ± 0.03 ml/g in liver and 0.33 ± 0.04 ml/g in kidney (mean of 8 experiments). The extracellular space for control liver and kidney slices (6) were found to be the same as shock liver and kidney slices. Thus with shock, there was no change in extracellular space. It is known that inulin distribution by liver reaches a steady-state level within 10–15 min and is maintained at least through 1 h (25). Increases in inulin uptake beyond 1 h have been interpreted as due to inulin binding to the tissues (25).

Penetration of ATP into shock liver and kidney. When liver and kidney slices from animals subjected to severe hemorrhage were incubated for 1 h in a medium containing 5 mM [^{14}C]ATP, extensive degradation of the nucleotide occurred (Figs. 1*A* and 2*A*). The principal breakdown products appearing in the medium at the end of the incubation period were inosine and adenosine. Whereas 0.07 μmol/ml ATP and ADP were detected in the medium containing liver slices only trace amounts of the above were detected in the medium containing kidney slices. This indicates that added ATP was degraded more by kidney than by liver.

As in control tissues (6), both inosine and adenosine have entered the liver and kidney cells of shock tissues (Figs. 1*A* and 2*A*). In control liver and kidney slices, intracellular inosine concentrations were found to be 0.36 ± 0.03 and 0.45 ± 0.04 μmol/*g*, respectively (6). The amount of inosine found intracellularly was approximately three times greater in shock tissues than in control tissues. In addition, externally added ATP was present intracellularly in shock tissues and amounted to at least 2.5 times the ATP found in controls. Intracellular ATP values in control liver and kidney slices were found to be 0.22 ± 0.02 and 0.14 ± 0.01 μmol/g, respectively (6).

When an ATP-regenerating system (phospho*enol*pyruvate: pyruvate kinase) was added to the medium during incubation (Figs. 1*B* and 2*B*), about 65% of the added ATP in the presence of shock liver and about 60% of the added ATP in the presence of shock kidney remained at the end of the incubation period. Under these conditions, intracellular ATP in both liver and kidney was approximately 2.5 times greater than without an ATP-regenerating system. In the presence of an ATP-regenerating system, although intracellular ATP was greater, intracellular ADP was decreased.

ADP as a substrate. When ^{14}C-labeled ADP was added to the incubation medium containing tissues from animals subjected to shock, extensive degradation of the added nucleotide occurred (Figs. 1*C* and 2*C*). As in control tissues (6), one of the products of ADP breakdown was ATP, produced by the action of adenylate kinase. Both ATP and ADP were found intracellularly

in approximately equal amounts. The uptake of ADP by shock tissues was the same as the uptake by control tissues (6). Thus with shock, there was no enhancement of ADP uptake.

AMP as a substrate. Extensive degradation of ^{14}C-labeled AMP was observed when this nucleotide was added to the incubation medium containing liver and kidney slices of animals subjected to shock. However, as with control tissues (6), externally added AMP was restricted to the extracellular environment in shock tissues.

Adenosine as a substrate. When adenosine was used as a substrate, degradation of this nucleotide also occurred (Figs. 1*D* and 2*D*). Intracellular adenosine was found to be the same in shock tissues as in control tissues (6). However, in control tissues, approximately 9% of the adenosine was synthesized to ATP whereas in shock tissues, labeled intracellular ATP was not detected in either the liver or the kidney. Intracellular AMP in shock liver and kidney was found to be 0.07 and 0.04 μmol/g as compared to 0.02 and 0.01 μmol/g in control liver and kidney when adenosine was used as a substrate.

DISCUSSION

It is a popular belief that cell membranes are impermeable to ATP (10) but many experimental observations indicate the contrary. Forrester (9) showed that ATP was released from active skeletal muscle under conditions in which K^+ was not. Silinsky and Hubbard (22) found that ATP is released from motor nerve terminal on indirect stimulation of a mammalian nerve-muscle preparation. We have shown that ATP entered intact skeletal muscle cells (1), and liver and kidney cells and that this process may be a carrier-mediated process (6). From the above-mentioned studies (1, 6, 9, 22), it may be concluded that the release and uptake of ATP is a physiological process. The present study was undertaken to determine if ATP uptake by tissues is altered during hemorrhagic shock.

In this study, we have proposed that the distribution of ^{14}C-labeled ATP between medium and tissue, shown in Figs. 1 and 2, is an indication that ATP added externally has entered the liver and kidney cells. It is appropriate, however, to discuss the intracellular distribution of radioactive ATP, the question as to whether the labeled ATP entered the cells as ATP, or whether it could have been synthesized from adenosine. The results presented in this paper are based on the assumption that the concentration of isotopically labeled nucleotide in the extracellular water is the same as that in the external medium. On the basis of the concentration in the medium shown in Figs. 1 and 2, it is possible to calculate the amount of radioactive nucleotide found in the extracellular space. If the amount of radioactive nucleotide found in the tissue exceeds the extracellular value, one may assume either that this excess is present intracellularly, or that there is an accumulation of the nucleotide in the extracellular space. Because of the extensive degradation of external ATP, it is difficult to visualize that the observed accumulation of ATP occurred in the extracellular space or on the surface of the cells.

It is known that some substances such as phlorizin and certain hormones which bind to cell membranes without entering the cells have larger distribution volumes than extracellular water. It is unlikely, however, that the same is true for ATP due to the fact that *1*) extensive degradation of external ATP occurred when tissues were present in the medium; *2*) the volume of distribution of ADP was much less than that of ATP, and *3*) external AMP failed to have an intracellular distribution.

In this study, we found a nearly complete absence of ATP in the medium when this nucleotide was added to a medium containing liver and kidney slices from animals subjected to hemorrhagic shock. In control tissues (6), despite an extensive degradation, there still was some ATP left in the medium at the end of the incubation (Figs. 1*A* and 2*A*). Thus, it may be concluded that the degradation of ATP is greater in the presence of shock tissues than in control tissues. This could be due to the increased ATPase activity during shock (8, 26). The absence of appreciable amounts of medium ATP in the present studies following incubation as compared to mediums containing control slices (6) could also be due to the fact that more ATP is taken up by shock than control tissues. After 1 h of incubation of shock tissues in the presence of ATP, intracellular ^{14}C-labeled ATP was found to be 0.55 μmol/g in liver and 0.38 μmol/g in kidney. These intracellular values were 2.5 times greater than the corresponding values in normal tissues (6). Thus with shock, there is an increase in the ATP uptake by tissues from external medium. The ratio of ATP and ADP was approximately 2 in both tissues when ATP was used as a substrate.

The present study demonstrates that after 1 h 0.55 μmol/g ATP in liver and 0.38 μmol/g ATP in kidney were present as [^{14}C] ATP when slices from these organs were incubated in the presence of [^{14}C] ATP (Figs. 1*A* and *2*). In a static system, this would suggest that only 11% and 7.6% of the added ATP was taken up by the liver and kidney, respectively, as ATP. However, since intracellular ATP is subjected to utilization for reactions in various intracellular energy-requiring processes, more ATP could have entered the tissue cells from the external medium and be subjected to utilization and hence degradation. The finding that intracellular adenosine concentration was the same irrespective of whether 5 mM ATP (without regenerating system) or 5 mM adenosine was used as a substrate strongly suggests that a certain amount of labeled ATP taken up by liver and kidney was indeed subjected to intracellular utilization-degradation. Thus, the intracellular radioactive ATP values at the end of the incubation period may not be a true representation of the total amount of ATP taken up by the tissues from the medium. Although the above experiments have shown that more ATP is taken up by liver and kidney of animals in shock, the subcellular distribution of ATP within the preparation into which the nucleotide entered is not known at the present time.

Since intracellular ATP and ADP are interconverti-

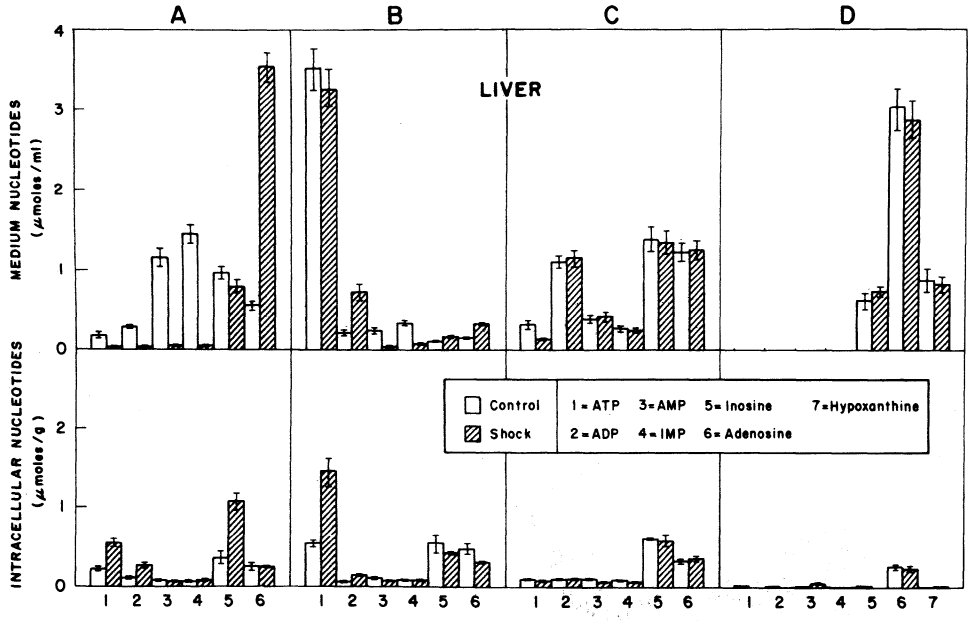

FIG. 1. Concentration of labeled nucleotide in the medium and tissue after rat liver slices from control animals and from animals in shock were incubated for 1 h at 37°C under 95% O_2-5%CO_2 in 1.0 ml of glucose-bicarbonate medium containing: *A*, 5 mM [^{14}C]ATP (0.45 μCi/μmol) and 5 mM $MgCl_2$; *B*, 5 mM [^{14}C]ATP (0.45 μCi/μmol), 5 mM $MgCl_2$, 20 μmol phospho*enol*pyruvate, 4 units pyruvate kinase (1 unit = the amount of enzyme which phosphorylates 1 μmol ADP/min at 37°C); *C*, 5 mM [^{14}C]ADP (0.25 μCi/μmol) and 5 mM $MgCl_2$; or *D*, 5 mM [^{14}C]adenosine (2 μCi/μmol) and 5 mM $MgCl_2$. Radioactivity present as adenine and hypoxanthine nucleotides in medium and tissue was counted following electrophoretic separation. Intracellular nucleotides were calculated as described under METHODS. Height of each column represents mean value of 8 animals ± SE.

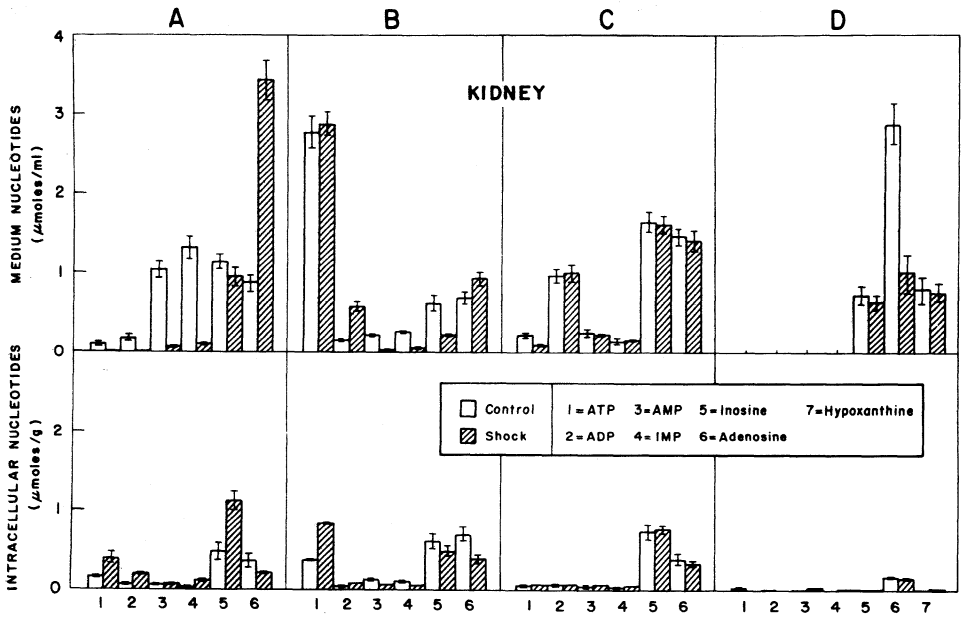

FIG. 2. Concentration of labeled nucleotide in the medium and tissue after rat kidney slices from control animals and from animals in shock were incubated for 1 h at 37°C under 95% O_2-5% CO_2 in 1.0 ml of glucose-bicarbonate medium containing: *A*, 5 mM [^{14}C]ATP (0.45 μCi/μmol) and 5 mM $MgCl_2$; *B*, 5 mM [^{14}C]ATP (0.45 μCi/μmol) 5 mM $MgCl_2$, 20 μmol phospho*enol*pyruvate, 4 units pyruvate kinase (1 unit = the amount of enzyme which phosphorylates 1 μmol ADP/min at 37°C); *C*, 5 mM [^{14}C]ADP (0.25 μCi/μmol) and 5 mM $MgCl_2$; or *D*, 5 mM [^{14}C]adenosine (2 μCi/μmol) and 5 mM $MgCl_2$. Radioactivity present as adenine and hypoxanthine nucleotides in medium and tissue was counted following electrophoretic separation. Intracellular nucleotides were calculated as described under METHODS. Height of each column represents mean value of 8 animals ± SE.

ble and since more intracellular ADP was detected in shock tissues than in control tissues, (6) it was not clear whether the radioactive ATP and ADP within liver and kidney cells of animals subjected to shock had entered as ATP or ADP, or was synthesized within the cell from adenosine or inosine. These questions were resolved by experiments using *1*) an ATP-regenerating system, *2*) ^{14}C-labeled ADP, *and 3*) ^{14}C-labeled adenosine.

When an ATP-regenerating system was used to maintain a high ATP:ADP ratio in the medium, intracellular ATP in liver and kidney was found to be 2.5 times greater than without the regenerating system. It should be noted that although intracellular ATP in shock tissues was 2.5 times greater with an ATP-regenerating system, this amount was actually 5 times greater than that of the control tissues without the regenerating system (6). With the ATP-regenerating system, not only was there much more intracellular ATP in shock tissues, but the ratio of ATP to ADP also increased to 10 in both tissues.

When ADP was used as a substrate, degradation of this nucleotide also occurred. One of the products of ADP degradation in the medium was a trace amount of ATP which was probably formed through the action of adenylate kinase. Adenylate kinase normally resides in the space between the two membranes of the mitochondria. Since a trace amount of ATP was formed in the medium when ADP was used as a substrate, this might indicate the presence of broken cells and damaged mitochondria in the present study. However, when an intact soleus muscle was incubated in the presence of 5 mM ADP, the amount of ATP formed under those conditions (1) was the same as in the present study. Thus, since adenylate kinase activity was also demonstrable when an intact muscle preparation was used, it is unlikely that the formation of ATP from ADP in the present

study is due to broken cells and damaged mitochondria. On the contrary, the presence of ATP in the medium in this study when ADP was used as a substrate might indicate that ADP was transported into the cell and mitochondria where it is subjected to the adenylate kinase reaction. Some of the ATP formed stays within the cell as demonstrated by the presence of intracellular labeled ATP while some leaves the cells and appears in the medium.

Using ADP as a substrate, there was some intracellular ATP but the ratio of ATP to ADP was 1 in both tissues. It was also found that the amounts of intracellular ATP and ADP in shock tissues were the same as the amounts in control tissues (6). Thus with shock, there was neither an increase in ADP uptake nor an increase in its conversion intracellularly to ATP. Although in the presence of ADP in the medium, there was some intracellular ATP, this value was approximately 3 times less than when ATP was used as a substrate. Thus even during shock, it is not possible that when ATP served as a substrate the ATP present intracellularly was due to synthesis from ADP. This together with the preponderance of ATP intracellularly when an ATP-regenerating system was used, clearly indicates that even during shock ATP entered liver and kidney as such and was not synthesized from ADP.

When ^{14}C-labeled adenosine was used as a substrate, ATP was not detected intracellularly in either the liver or the kidney of animals in shock. This contrasts with control tissues in which some ATP synthesis took place from adenosine (6). There was an increase in the amount of intracellular AMP in both liver and kidney from animals in shock, suggesting that the synthetic pathway might be blocked at the level of AMP in shock tissues. We have previously proposed that the decreased levels of ATP during shock may well be due to difficulty in production rather than accelerated utilization (4). The present results support our previous hypothesis and suggest that the ATP resynthesis problem during shock may be in the area of AMP, i.e., its conversion to ADP.

Relating the present results to our previous studies (3), one would expect a beneficial effect from ATP but not from ADP administered to animals in shock, since in shock tissues more ATP but not ADP entered the cells. We have previously shown that infusion of ATP-$MgCl_2$ but not ADP-, AMP-, or adenosine-$MgCl_2$ proved beneficial in the treatment of animals in shock (3).

The present study has demonstrated that under the altered cellular environment found in shock more ATP enters the liver and kidney cells as ATP to replenish the cells in which ATP levels were significantly lowered. We have also found that there is an increase in ATP uptake by skeletal muscle of animals in shock (5). The enhancement in ATP uptake by tissues during shock could be due to an increase in membrane permeability. Possible alterations in membrane permeability in tissues during shock were suggested by the recent work of Sayeed and Baue (20). Furthermore, supportive evidence for the beneficial effect of ATP during adverse conditions also suggests that alterations in membrane permeability occur. The work of Horska et al. (11) has shown that by administering ATP to the mother during delivery fetal hypoxia was relieved with respect to both metabolic and gaseous exchange. They also showed that the metabolic improvement of the fetus during delivery was reflected by the more favorable condition of the infants at birth. The studies of Ziegelhoffer et al. (27) have shown that administration of ATP in isolated hypoxic and hypothermic dog hearts resulted in increased tissue contents of ATP and the total adenine nucleotides. In the above studies (11, 27) as well as in the present study, it is likely that under adverse conditions such as are found in shock and hypoxia more ATP must cross the cell membrane. Relating the present finding to our previous studies (3), it may be concluded that the beneficial effect of ATP-$MgCl_2$ infusion to animals in shock could be through direct provision of energy to cells in which ATP levels were lowered.

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