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Uncoupling Agents Distinguish Between the Effects of Metabolic Inhibitors and Transport Inhibitors

Abstract. *In studies with toad bladders, the uncoupling agent 2,4-dinitrophenol (DNP) reversed the inhibition of CO_2 production produced by direct inhibition of transport. In contrast, DNP did not reverse the inhibition of CO_2 production brought about by metabolic inhibitors. Therefore, the response to DNP distinguished between inhibition of transport and metabolism; this approach may be useful for the investigation of factors that regulate active transport.*

In many biological systems the processes of energy production by metabolic pathways and energy utilization vary in a parallel fashion—that is, metabolism and biological work are tightly coupled. This is because the rate of oxidative phosphorylation (and glycolysis) is regulated by the availability of adenosine diphosphate (ADP), a phenomenon termed "respiratory control" (*1*). The close relationship between energy production and energy utilization has made it difficult to elucidate the mechanism of action of factors that influence these processes. In the case of active transport, the hormones aldosterone and thyroxine have been shown to stimulate metabolism (*2*) and transport (*3*). Similarly, diuretic drugs have been shown to inhibit metabolism (*4*) and transport (*5*). Because metabolism and transport are tightly coupled, the primary mechanism of action of these agents remains controversial.

In the present experiments, the uncoupling agent 2,4-dinitrophenol (DNP) was used to distinguish between agents that directly inhibit transport and agents that directly inhibit metabolism. Uncoupling agents have the ability to stimulate respiration when ADP is rate-limiting (*6*). Therefore, if active transport is directly inhibited, respiration would be diminished secondarily, because of reduced adenosine triphosphate (ATP) utilization and ADP production; in this case, DNP would be expected to restimulate respiration. In contrast, if metabolism is directly inhibited, DNP would not be expected to restimulate respiration.

Experiments were performed with urinary bladders of toads (*Bufo marinus*) obtained from the Dominican Republic. Hemibladders were mounted in chambers and continuously voltage clamped. The short-circuit current (SCC) of these bladders is an accurate measurement of active sodium transport (*7*). The rate of CO_2 production (QCO_2) by the bladder was continuously monitored by a method using differential conductivity (*8*). Each bladder was divided into an experimental and a control hemibladder. The SCC and QCO_2 of control hemibladders declined slightly during the 2-hour experiment. After a 1-hour baseline measurement period, an inhibitor was added to the experimental hemibladder. Continuous monitoring of SCC and QCO_2 indicated that a new steady state was reached in 45 to 60 minutes. At this time $10^{-5}M$ DNP (shown in dose-response experiments to be maximally effective) was added to both the control and experimental hemibladders. The peak QCO_2 after the addition of DNP was then recorded.

The results are shown in Table 1. Direct inhibition of active transport either by removal of sodium from the bathing solutions (and substitution with choline) or by the addition of $10^{-3}M$ ouabain (which markedly inhibits active sodium transport) significantly inhibited the SCC and lowered the QCO_2. The subsequent

Table 1. Effects of transport and metabolic inhibitors and DNP on QCO_2 of toad bladders. The data are expressed as means ± standard error. The short-circuit current (SCC) was measured in microamperes; CO_2 production (QCO_2) was measured in microliters per hour. Na^+ removal was accomplished by substituting choline in the Ringer solutions for Na^+. The *P* values indicate whether the change in QCO_2 produced by DNP was significant; NS, not significant.

Type of inhibition	Baseline values		After inhibition		After addition of $10^{-5}M$ DNP		Change produced by DNP	
	SCC	QCO_2	SCC	QCO_2	SCC	QCO_2	QCO_2	*P*
Inhibition of transport								
Na^+ removal ($N = 8$)*	170 ± 34	22 ± 3	7 ± 7	12 ± 2	5 ± 5	23 ± 3	11	<.01
Ouabain ($1 \times 10^{-3}M$) ($N = 13$)	203 ± 54	40 ± 4	75 ± 59	33 ± 4	2 ± 1	78 ± 8	45	<.001
Inhibition of metabolism								
Antimycin A ($1 \times 10^{-5}M$) ($N = 9$)	185 ± 38	31 ± 7	46 ± 9	16 ± 6	15 ± 5	16 ± 6	0	NS
Rotenone ($1 \times 10^{-5}M$) ($N = 10$)	144 ± 26	22 ± 2	55 ± 8	12 ± 1	20 ± 3	13 ± 2	1.0	NS

*N is the number of hemibladders.

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addition of $10^{-5}M$ DNP further inhibited SCC, but the QCO_2 was significantly increased. This effect is consistent with the action of an uncoupling agent which diminishes ATP production while stimulating metabolism by removing the rate-limiting constraints of respiratory control. The respiratory inhibitors rotenone and antimycin (*9*) also inhibited SCC and QCO_2. In marked contrast to the transport inhibitors, DNP had no significant effect on QCO_2 in the presence of rotenone or antimycin. Although CO_2 may be produced by the pentose shunt pathway, the respiratory quotient of toad bladders is constant (*10*); thus, it is unlikely that changes in the rate of the pentose shunt pathway could account for the present findings.

These results are consistent with the view that respiration which is diminished by direct inhibition of transport can be restimulated by the presence of an uncoupling agent; direct inhibition of respiration by a metabolic inhibitor cannot be reversed by an uncoupling agent. This approach may be useful in the investigation of physiological factors or pharmacological agents that affect biological work functions.

MICHAEL W. WEINER
Department of Medicine,
Stanford University School of Medicine,
Veterans Administration Hospital,
Palo Alto, California 94304

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Liposomes and Local Hyperthermia: Selective Delivery of Methotrexate to Heated Tumors

Abstract. *Liposomes with phase transitions a few degrees above physiological temperature delivered more than four times as much methotrexate to murine tumors heated to 42°C as to unheated control tumors. Most of the accumulated drug appeared to be intracellular and bound to dihydrofolate reductase, the enzyme blocked by methotrexate in its role as an antineoplastic agent.*

Systemic drug therapy is a notoriously blunt weapon with which to attack local disease. In a few cases, for instance with antibiotics, a drug may be so selective in action that therapeutic concentrations can be achieved without toxic side effects. More often, a risk of harmful effects must be tolerated. In tumor chemotherapy, for example, the beneficial and toxic actions are so delicately balanced that relatively small degrees of selective drug localization could be useful. We recently suggested a way of combining liposomes as drug carriers with local hyperthermia to achieve preferential local release of drug in a target area (*1*). We now present experiments clearly showing such an effect in vivo.

Liposomes (*2*) are microscopic particles consisting of a single lipid bilayer enclosing a single aqueous compartment (unilamellar vesicle) or a number of concentric bilayers enclosing an equal number of aqueous spaces (multilamellar vesicle). Their use as pharmacological capsules has been limited largely by inability to direct them to particular cells or anatomical sites. Local hyperthermia (*3*), the heating of a region of the body a few degrees above its normal temperature, may provide a basis for such selectivity. Hyperthermia is under study for tumor therapy because it has been reported to affect tumor cells more than normal cells (*4*) and to have synergistic actions in combination with drugs (*5*) and with radiotherapy (*6*).

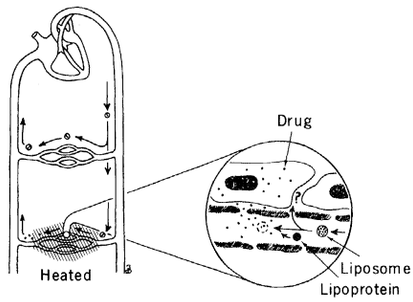

Fig. 1. Schematic view of hypothetical preferential release of liposome contents in a heated area. As liposomes enter the small vessels of the heated region they release their contents at a rate dependent on temperature, the rate of change of temperature, and the action of serum components. Released drug equilibrates throughout the extracellular space and is transported into cells as if injected in free form. As indicated by the question mark, intact liposomes might also pass into the extracellular space through endothelia made leaky by heating, and lipid molecules might be exchanged with cells directly or through the mediation of serum components such as lipoproteins.

Our approach to the combination of liposomes with hyperthermia is shown schematically in Fig. 1 for the case of intravenous injection. Lipids are selected so as to make liposomes with liquid-crystalline phase transition temperatures (T_c) a few degrees above physiological, near the range obtainable by local hyperthermia. The liposomes remain relatively stable in the circulation at temperatures well below T_c but release their contents as T_c is approached (*7*). The rate of release is dependent on the rate of change of temperature and is markedly enhanced by serum components (*1*), principally lipoproteins (*8*). Essentially total release of liposome contents can be achieved in vitro within a few seconds by raising the temperature through T_c in the presence of serum, so it seemed possible that large fractional releases could be achieved in vivo during passage of liposomes through the small arteries, arterioles, and capillaries of a heated region. But it was not clear that conditions of local hyperthermia safe for normal tissues (for example, heating to 42°C for 1 hour) could be exploited effectively. It also seemed possible that the release might take place for the most part in the venous system, from which the drug would simply be washed out of the area by blood flow.

Our aim, therefore, was to develop an experimental system in which the principles of this approach could be studied quantitatively. We chose to use the drug methotrexate (MTX) for three reasons. First, its sodium salt is highly water-soluble and can therefore be encapsulated in and released from liposomes. Second, it acts by binding tightly to a cytoplasmic enzyme, dihydrofolate reductase, with a binding constant of approximately $10^{10}M^{-1}$ (*9*). Therefore, as long as the enzyme's binding capacity is not exceeded, MTX will remain bound in cells long after blood levels have largely dissipated, permitting measurement of total drug de-

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