EFECTO DE LA ADENOSINA SOBRE EL HIGADO GRASO PRODUCIDO POR ETANOL, CICLOHEXIMIDA, TETRACLORURO DE CARBONO Y ETIONINA.

T E S I S

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RESUMEN:

La administración intraperitoneal de adenosina evita (parcial o totalmente la inducción de hígado graso por etanol, ciclohexímida, tetracloruro de carbono y etionina. El mecanismo general por el cual se produce este efecto hepatoprotector por el nucleósido es una inhibición del metabolismo hepático de los ácidos grasos. Otros mecanismos de acción participan en el fenómeno general; así, en el caso del daño producipo por el metabolismo del etanol se observó que un aumento en la utilización de equivalentes reductores (NADH) participa en la acción del nuclósido. Este aumento en la utilización de equivalentes reductores parece deberse a una modificación en la cadena respiratoria que se observa preferentemente a nivel del sitio I.

INTRODUCCION

Uno de los grandes problemas de salud es la cirrosis hepática. Esta enfermedad pudiera ser definida como "un aumento difuso, real o aparente de tejido conectivo en el hígado, por lo regular acompañado de necrosis y regeneración de la células parenquimatosas, que dan al hígado un aspecto nodular o granular" (1). Existen múltiples causas de cirrosis hepática: alcoholismo, alteraciones en el flujo biliar, hemocromatosis, infecciones virales, etc. Sin embargo, la causa mas frecuente es la exposición crónica a agentes químicos tóxicos, como son el etanol, el tetracloruro de carbono, el cloroformo y otros disolventes orgánicos empleados frecuentemente. Es conocida la asociación entre alcoholismo y cirrosis hepática. Durante muchos años, dada la complejidad de los problemas metabólicos que acarrea el alcoholismo, se ha sostenido la idea de que el consumo de alcohol solo conduce a daño hepático cuando se asocia a una dieta deficiente. Lieber y colaboradores (2) han demostrado en primates que la ingesta crónica de etanol produce daño hepático (hígado graso, hepatitis alcohólica y cirrosis en forma secuencial) a pesar de una dieta adecuada.

La producción de cirrosis hepática es un proceso largo y que tanto desde el punto de vista patológico como bioquímico está lleno de interrogantes no resueltas. Se ha observado tanto clínica como experimentalmente, que el daño usualmente se inicia con una acumulación de triacilglicéridos en el hepatocito (proceso que llamamos hígado graso; nota: algunos autores de nuestra lengua prefieren otras denominaciones como son: hígado grasoso, hepatoesteatosis, lipósis del hígado, degeneración grasa del hígado, etc. Dado que un cambio en la denominación del cuadro no es de utilidad científica alguna, dejo a los lectores de esta tesis el cambiar hígado graso por el término de su agrado). Esta acumulación de triacilglicéridos en hígado puede evolucionar favorablemente y desaparecer o pasar a la etapa de hepatitis alcohólica (caracterizada por necrosis). Los factores que determinan la transición no son conocidos y no existe una explicación bioquímica satisfactoria hasta el momento.

c)En los estudios con etanol se ampliaron las exploraciones evaluandose además los estados energético y de oxido-reducción (citoplásmico y mitocondrial) y la capacidad oxidativa mitocondrial.

RESUMEN DE RESULTADOS

Los resultados se encuentran en los trabajos que a continuación se presentan (referencias 11 a 15).

El resultado fundamental es que la administración de adenosina evita o revierte, parcial o totalmente, el hígado graso producido por etanol (11, tabla III; 12, Fig. 1), cicloheximida (11, tabla I; 14, Fig 1 y Tabla 1), etionina (11, tabla II) y tetracloruro de carbono (11, tabla IV).

Algo interesante y que nos indica la necesidad de comprobar cada punto de una hipótesis es lo siguiente: Los hepatotóxicos no tienen contra lo propuesto por Isselbacher (6), como mecanismo general de acción el abatimiento de los niveles energéticos de la célula. Esto se observa claramente en el caso de intoxicación etílica (12, tablas IV y V). La administración de etanol altera poco los parámetros energéticos de la célula hepática.

Quedamos por lo tanto con un efecto y la necesidad de darle una explicación bioquímica. En el caso de la intoxicación por etanol, es bien sabido, que las alteraciones metabólicas que ocasiona, son debidas a su metabolismo, es decir, a la generación de equivalentes reductores (NADH). Al explorar el efecto de la adenosina sobre el estado redox citoplásmico se observo que carecía de efecto en condiciones basales pero cuando se administraba junto con etanol, el nucleósido revertía parcialmente el efecto del etanol.

Existía por lo tanto la posibilidad de que la adenosina inhibiera el metabolismo del etanol y asi ejerciera su efecto. Sin embargo, la velocidad de depuración de etanol se aumenta con adenosina (12, Fig. 3). Dado que es la reoxidación del NADH el paso limitante para la oxidación del etanol, nuestros resultados indican que la administración de adenosina aumenta la capacidad oxidativa del

La etapa de hepatitis suele ser breve y evolucionar hacia cirrosis. Es importante enfatizar en este momento que la etapa de hígado graso es la etapa reversible del proceso y que los esfuerzos para detener el avance de la enfermedad se deben realizar en ese estado.

Farber y colaboradores (3-5) han demostrado la importancia del estado energético del hepatocito en la producción de hígado graso por etionina. Isselbacher (6) ha señalado que una característica general de los agentes que producen hígado graso, es disminuir el estado energético de la celdilla hepática. En su publicación, este autor indica que la administración de ATP, mejora el estado energético y revierte o mejora el hígado graso; sugiere además una relación causal entre ambos procesos, i. e., la caída en estado energético conduce a la acumulación de tiriacilglicéridos.

Chagoya de Sánchez, Brunner y Piña (7) reportaron que la administración de adenosina a ratas, eleva considerablemente los parámetros energéticos de la celdilla hepática. Esta observación ha sido confirmada en otros sistemas como son el hígado perfundido (8), las rebanadas de hígado (9) y los hepatocitos aislados (10). De tal suerte, que la adenosina se ha convertido en un arma metabólica para incrementar el estado energético del hepatocito.

Tomando en cuenta que en la génesis de cirrosis, la etapa reversible es la de hígado graso y dada la relación "caida de la carga energética---→ inducción de hígado graso (6), se estableció la siguiente hipótesis de trabajo: la administración de adenosina (que eleva los parametros energéticos hepáticos) probablemente evite la inducción de hígado graso. Se decidió explorar los siguientes aspectos:

a) Efecto de la adenosina sobre el hígado graso producido por los siguiente agentes: etanol, etionina, cicloheximida y tetracloruro de carbono. Estos agentes fueron seleccionados por ser los mas ampliamente usados y de los que tenemos mas información acerca de sus acciones y mecanismos de acción.

b) Estudio de la dinámica del metabolismo de los lípidos para conocer el mecanismo de acción del hepatotóxico y de la adenosina

hepatocito (el mecanismo por el cual esto ocurre fue explorado y los datos se comentan más adelante). Establecimos estadísticamente la relación entre hígado graso y estado redox citoplásmico (12, Fig. 2) la cual fue casi perfecta con un coeficiente de correlación r= 0.99

Debe recordarse que correlación no significa causalidad. Es poco probable que la adenosina antagonizara específicamente las acciones de los hepatotóxicos y que no existiera un mecanismo común de acción en el efecto hepatoprotector del nucleósido. El hecho de que la adenosina evita la producción de hígado graso por agentes que no actuan a través de modificar el estado redox citoplasmico (como cicloheximida, etionina o tetracloruro), nos sugería la existencia de por lo menos otro mecanismo de acción.

Para estudiar el mecanismo general de acción del nucleósido fueron de gran utilidad los experimentos con alopurinol y cicloheximida. Cuando tratamos a las ratas con alopurinol (inhibidor de la xantin-oxidasa) observamos que ahora la adenosina era capaz de evitar totalmente el hígado graso producido por etanol (en ausencia de alopurinol la acción era parcial), pero solo parcialmente las alteraciones en el estado redox (13, ver texto) lo cual rompe la correlación entre ambos procesos (r= 0.56). Al estudiar la dinámica lipídica se observó que la administración de adenosina y etanol en presencia de alopurinol producía una elevación significativa en los niveles séricos de ácidos grasos libres (13, ver texto). La concentración de ácidos grasos es proporcional a su metabolismo hepático. Sabiendo que la administración de adenosina disminuye los niveles de cuerpos cetónico (oxidación) y los niveles séricos y hepáticos de triacilglicéridos (esterificación) resulta evidente que una disminución general en el metabolismo de los ácidos grasos por el hígado pudiera ser un factor responsable del effecto hepatoprotector del nucleósido. Esta sugerencia es consistente con la inhibición observada tanto <u>in vivo</u> como <u>in vitro</u> de la acil-CoA sintetasa (16,17) Vale la pena enfatizar en este momento, que la actividad de esta enzima es indispensable para el metabolismo de los ácidos grasos de cadena larga.En los expe-

rimentos con cicloheximida se comprobó este efecto del nucleósido midiendo la incorporación de palmitato radioactivo a lípidos hepáticos.

Algunos hallazgos interesantes se lograron también, usando cicloheximida como hepatotóxico. Comprobamos el efecto del antibiótico sobre el nivel sérico de ácidos grasos libres, que se debe a su acción sobre tejido adiposo aumentando la esterificación (18-20) y se encontro que el antibiótico tambien aumenta la esterificación hepática de los ácidos grasos disminuyendo su flujo hacia oxidación.

Tratando de pro undizar sobre la acción de la adenosina sobre el metabolismo oxidativo se decidió estudiar la función mitocondrial en hígado de animales tratados con el nucleósido. Se observó que la administración del nucleósido incrementa la respiración mitocondrial y que este efecto probablemente se deba a alguna alteración en la cadena respiratoria ya que persiste el efecto en partículas submitocondriales.

CONCLUSIONES

a) La administración de adenosina evita la producción de hígado graso (parcial o totalmente) por etanol, cicloheximida, etionina y tetracloruro de carbono.

b) El nucleósido parece ejercer esta acción hepatoprotectora por un mecanismo general: disminuir el metabolismo hepático de los ácidos grasos. Otras acciones del nucleósido pudieran estar involucradas en casos especiales: i.e., en el caso de la intoxicación etílica, por ejemplo.

c) Se comprobó indirectamente la importancia de la movilización de ácidos grasos de los depósitos en la génesis de hígado graso.

d) La administración del nucleósido ejerce una estimulación sobre el metabolismo oxidativo mitocondrial que involucra un incremento en la capacidad respiratoria mitocondrial.

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EFECTO DE LA ADENOSINA SOBRE EL HÍGADO GRASO PRODUCIDO POR DIVERSOS HEPATOTÓXICOS

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El contenido normal de grasa en el hígado es de 4 a 5%, constituida de triglicéridos, ácidos grasos, colesterol y fosfolipidos. En condiciones de funcionamiento hepático normal los componentes lipídicos se encuentran en proporciones constantes. La capacidad de almacenamiento de lipidos en el hígado es limitada y está sujeta a una regulación muy estricta.

El nivel de grasa hepática es la resultante de varios factores, entre ellos señalaremos: a) biosíntesis de grasa, b) utilización para obtener energía, c) transporte de lípidos de los depósitos al hígado y d) movilización de lípidos del hígado a los depósitos. Estos factores se encuentran en equilibrio, de tal manera que los niveles de grasa hepática se mantengan dentro de los límites normales: cualquier condición que altere este equilibrio en el sentido de aumentar la formación de lípidos en el hígado, disminuir su utilización, estimular su movilización de los depósitos o impedir el transporte del hígado a los depósitos resultará en un aumento de lípidos en el hígado produciéndose una alteración metabólica conocida como hígado graso, también llamada hepatoesteatosis o degeneración grasa del hígado.

El hígado graso puede ser de dos tipos, fisiológico y patológico. El primero se caracteriza por mantener el patrón de componentes lipídicos normal y por ser fácilmente reversible; el segundo, tiene un patrón lipídico anormal presentando un aumento en la proporción de triglicéridos y ésteres de colesterol. Y puede degenerar en un daño hepático más severo y eventualmente a cirrosis hepática. Este último tipo de hígado graso es el que discutiremos en este trabajo.

La frecuencia de daño hepático aumenta continuamente como una consecuencia de factores socioculturales. Entre las situaciones clínicas más frecuentes que se asocian a este padecimiento tenemos: la desnutrición, especialmente en niños que reciben dietas normocalóricas hipoproteicas, consumo sin control de agentes terapéuticos, como antidepresivos, hipotensores y tetraciclinas [1,2,3], manejo de solventes orgánicos, alcoholismo, etc.

La correlación hígado graso-cirrosis no ha sido bien establecida [4,5] puesto que en muchos casos el hígado graso de tipo patológico también puede revertir sin que evolucione a cirrosis. Se sabe desde hace mucho tiempo que la ingestión de alcohol etílico, el más popular de los hepatotóxicos, conduce a la hepatoesteatosis y se ha considerado necesaria la concomitancia del alcoholismo y la desnutrición para que se produzca la cirrosis hepática. Sin embargo, hace algunos años Lieber, De Carli y Rubin [6] demostraron en primates que a pesar de una dieta adecuada, la administración crónica de etanol, es capaz de producir en forma secuencial hígado graso, hepatitis y cirrosis.

La cirrosis hepática es un problema de Salud Pública no sólo en nuestro país, donde ocupa la primera causa de muerte entre hombres de 40 a 59 años y la quinta entre hombres de 15 a 39 años [7] sino en todo el mundo. Bástenos mencionar que en la ciudad de Nueva York, es la tercera causa de muerte entre personas de 25 a 65 años de edad [8].

El daño hepático principia en diferentes regiones del hígado y por diversos mecanismos, en sus fases iniciales puede ser reversible, posibilidad que se va perdiendo a medida que aumenta el deterioro celular, por lo que se debe poner especial interés en el tratamiento de las fases reversibles del proceso.

Dado que los hepatotóxicos poseen diversos mecanismos de acción, no ha sido posible establecer una secuencia fisiopatológica del hígado graso. Sin embargo una de las primeras alteraciones metabólicas en este padecimiento es una caída importante en los niveles hepáticos de ATP [9] probablemente por una mayor utilización de energía y/o por interferencia en los procesos generadores de energía. Esta situación de alarma obliga a la economía celular a utilizar sus reservas: se produce una marcada desaparición del glucógeno hepático [9] y movilización de los ácidos grasos de los depósitos, hacia el hígado [10]. Algunos hepatotóxicos interfieren en la oxidación hepática de los ácidos grasos [11] lo cual ocasiona que sean convertidos en glicéridos y se acumulen. Simultáneamente al incremento catabólico los procesos anabólicos disminuyen: como la bio-

síntesis de purinas [12], ácidos nucleicos (RNA) y proteínas [13,14,15]. La biosíntesis de proteínas es indispensable para la síntesis de lipoproteínas que van a encargarse de la salida de los lípidos del hígado. Existe un número importante de condiciones que favorecen la formación del hígado graso, en que la síntesis de proteínas es deficiente, probablemente por aporte insuficiente de aminoácidos, por falta de energía o por algún bloqueo en el proceso de biosíntesis proteica.

Como todo mecanismo general, no es posible aplicarlo estrictamente a cada caso específico. Sin embargo pudiéramos tratar de integrar esta serie de observaciones en una secuencia lógica de eventos metabólicos:

1. Contacto con el agente causal.
2. Disminución de los procesos generadores de energía.
3. Caída de la carga energética (ATP).
4. Estímulo de los procesos catabólicos: a) glucogenólisis hepática, b) lipólisis periférica y movilización de ácidos grasos al hígado.
5. Inhibición de los procesos anabólicos: a) biosíntesis de purinas, b) biosíntesis de ácidos nucleicos, c) biosíntesis de proteínas, d) biosíntesis de glucógeno.
6. Acúmulo de lípidos en el hígado.

Antecedentes

Como resultado de las investigaciones realizadas en nuestro laboratorio, en relación a los efectos de la adenosina en el metabolismo intermedio, surgió la posibilidad de que pudiera ser de utilidad en el proceso antes descrito, es decir en el estado de hígado graso.

A continuación enumeraremos los efectos bioquímicos de la adenosina que nos impulsaron a usarlo en relación al hígado graso inducido por diversos hepatotóxicos:

1. Aumento del 40% en la poza de ATP, y consecuente elevación de la carga energética de la celdilla hepática [16].
2. Aumento de 8 veces de la actividad de la glucógeno sintetasa de hígado de rata [17].
3. Aumento de 13 veces la incorporación de glucosa ^{14}C al glucógeno hepático (glucogénesis) [18].

4. Aumento de 6 veces la incorporación de alanina ^{14}C al glucógeno hepático (gluconeogénesis-glucogénesis) [18].

5. Aumento de 5 veces de la lipogénesis en el tejido adiposo del epidídimo [18].

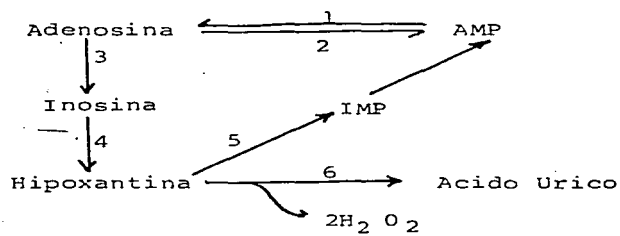
6. Acción antilipolítica de la adenosina *in vitro* [19].

7. Inhibición de la activación y oxidación de los ácidos grasos [20].

8. Disminución de la lipogénesis hepática [21].

Los cambios metabólicos que se obtienen por la administración de adenosina serían opuestos a los que observan en las primeras fases de hepatotoxidad ya que el efecto de la adenosina sería estimular los procesos biosintéticos e inhibir los procesos catabólicos [16].

Una ventaja adicional en el uso de la adenosina lo constituye el hecho de que este nucleósido es un metabolito normal en los tejidos; se ha reportado a la concentración de 1 a 2 μM en corazón, y cerebro [22,23] y 5 a 8 μM en hígado [24]. La adenosina tiene un recambio metabólico muy rápido [25] y lo haría a través de caminos enzimáticos ya existentes en la célula (Esquema 1), esto representa una ventaja al no usar los sistemas de desintoxicación del órgano dañado. La vía de desaminación del nucleósido (adenosina deaminasa) para la obtención de inosina, funciona activamente en el tejido hepático [26], no así la de fosforilación para la obtención de AMP (adenosina cinasa) [26]. En el tejido hepático existe una enzima muy relevante en el metabolismo de la purina (hipo-



ESQUEMA 1. Metabolismo de la Adenosina. 1, 5' Nucleotidasa. 2. Adenosina cinasa. 3. Adenosina deaminas. 4. Nucleósido fosforilasa. 5. Hipoxantina fosforibosil transferasa. 6. Xantino oxidasa.

xantina fosforribosil tranferasa)[26] que cataliza la tran-formación de hipoxantina a IMP, esta actividad corresponde al "camino de salvación de las purinas" o sea el camino de reutilización de los residuos de purina para regenerar el nucleótido de adenina. De los efectos de la adenosina que sirven como base a este proyecto discutiremos más ampliamente algunos de ellos.

Uno de los mecanismos generales postulado en la producción del hígado graso, es la caída de la carga energética[27,28]. nuestro grupo fue el primero en demostrar que la administración de adenosina es capaz de incrementar notablemente la carga energética del hígado[18]. Hace algunos años Farber y cols.[29] revirtieron el hígado graso producido por etionina con dosis grandes de ATP. Posteriormente. Hyams e Isselbacher[28] demostraron que el ATP es capaz de revertir el hígado graso producido por otros hepatotóxicos. Sin embargo, cuando el ATP es administrado a dosis moderadas, se recupera la carga energética pero no se evita el hígado graso[30], lo cual sugiere la participación de otros mecanismos.

Se ha observado que los triglicéridos acumulados en el hígado en la hepatoesteatosis son ricos en ácidos grasos esenciales y que su composición es similar a los del tejido adiposo[10,31]. Estos datos señalan que la movilización de los ácidos grasos de los depósitos (tejido adiposo) tiene un papel muy importante en la etiología del hígado graso. La adenosina es un inhibidor de la lipólisis en el tejido adiposo[18] y Fain, Pointer y Ward[32] han sugerido que la adenosina puede tener un papel regulador fisiológico de la adenilciclasa de tejido adiposo.

La acción esperada de la adenosina no está únicamente en la posible disminución de la afluencia de ácidos grasos de los depósitos al hígado. Para que éstos sean metabolizados, deben ser convertidos a ésteres de coenzima A por acción de la tiocinasa. Estudios recientes nos han demostrado que la adenosina es capaz de inhibir a esta enzima activadora de los ácidos grasos[20] impidiendo en esta forma la utilización de los ácidos grasos para la biosíntesis de triglicéridos y para su oxidación.

Resultados y discusión

Con estos datos en mente se inició una exploración general de la acción de la adenosina frente a varios agentes hepatotóxicos, con diferentes mecanismos de acción, a dosis y en tiempos recomendados en la literatura. Como sujeto experimental se usaron ratas machos de 130 a 200 gms. de peso corporal con un periodo de ayuno de 16 a 20 horas. Para valorar

el hígado graso se determinó la cantidad de triglicéridos hepáticos por el método de Butler, y cols. [33]. La adenosina se administró en suspensión en NaCl 0.85%, pH 7.4 a una dosis de 200 mg/Kg por vía intraperitoneal, óptima en nuestros estudios metabólicos previos.

Los hepatotóxicos probados fueron: la cicloheximida, la etionina, el etanol y el tetracloruro de carbono. La cicloheximida es un inhibidor de la síntesis de proteínas citoplásmicas, se postula que produce hígado graso, al bloquear la síntesis de las lipoproteínas, indispensables para la movilización de triglicéridos hepáticos [34,35]. Sin embargo este mecanismo como único responsable de la hepatoesteatosis inducida por cicloheximida se ha puesto en duda recientemente por Sabesin [36] quien demuestra, usando acetoxicicloheximida que la inhibición de la síntesis de proteína hepática no es suficiente para producir acumulación de triglicéridos en el hígado [36]. Por otra parte, en nuestro laboratorio hemos observado que la cicloheximida es capaz de incrementar el proceso de esterificación en tejido adiposo [37,38]; tal vez este mecanismo participe en el hígado. En nuestras condiciones experimentales la cicloheximida produce un incremento del 325% en la cantidad de triglicéridos hepáticos. La administración simultánea de adenosina evita este proceso (tabla I).

La etionina es un hepatotóxico que produce el hígado graso al depletar de ATP a la celdilla hepática, produciendo en forma secundaria una inhibición de la síntesis de proteínas [29]. En la tabla II, se aprecia el incremento en la cantidad de triglicéridos hepáticos que produce esta sustan-

TABLA I. EFECTO DE LA ADENOSINA EN LA ACUMULACION DE TRIGLICERIDOS PRODUCIDA POR LA ADMINISTRACION DE CICLOHEXIMIDA

Tratamiento	Nivel de Triglicéridos Hepáticos mg/g	p*	p**
Salina + salina (5)	2.99 ± 0.27		
Salina + adenosina (5)	2.28 ± 0.38	<0.2	
Cicloheximida + salina (5)	9.74 ± 1.04	<0.001	
Cicloheximida + adenosina (5)	3.30 ± 0.41	<0.6	<0.001

* vs. salina + salina: ** vs. cicloheximida + salina. Se administró cicloheximida a dosis de 3.3 mg/Kg intraperitoneal. Los animales se sacrificaron 4 horas después del tratamiento Los resultados se expresan como el promedio ± el error estándar del grupo.

TABLA II. EFECTO DE LA ADENOSINA EN LA ACUMULACION DE TRIGLICERIDOS PRODUCIDA POR LA ADMINISTRACION DE ETIONINA

Tratamiento	Nivel de Triglicéridos Hepáticos mg/g	p*	p**
Salina + salina (7)	2.44 ± 0.49		
Salina + adenosina (12)	3.75 ± 0.74	<0.2	
Etionina ± salina (10)	10.70 ± 2.66	<0.01	
Etionina + adenosina (10)	2.35 ± 0.44	<0.9	<0.01

* vs. salina + salina; ** vs etionina + salina. Se administró etionina a dosis de 1 g/kg intraperitoneal. Los animales se sacrificaron 5 horas después del tratamiento. Los datos se expresan como el promedio ± el error estándar del grupo.

cia y cómo es evitado por la administración simultánea de adenosina.

A pesar de ser el etanol el hepatotóxico más conocido y uno de los más estudiados, no se conoce el mecanismo exacto por el cual produce hígado graso. Actualmente se acepta que la avalancha de equivalentes reductores que produce su metabolismo es en gran parte responsable del daño que ocasiona (39). La administración de una dosis elevada de etanol produce un acúmulo de triglicéridos en el hígado, como puede apreciarse en la tabla III, la administración simultánea del nucleósido de la adenina revierte parcialmente el proceso.

El tetracloruro de carbono es un tóxico que actúa fundamentalmente peroxidando los lípidos microsomales (40). Los mecanismos más acepta-

TABLA III. EFECTO DE LA ADENOSINA SOBRE LA ACUMULACION DE TRIGLICERIDOS PRODUCIDA POR LA ADMINISTRACION DE ETANOL

Tratamiento	Nivel de Triglicéridos Hepáticos mg/g	p*	p**
Glucosa + salina (6)	2.69 ± 0.39		
Glucosa + adenosina (4)	3.09 ± 0.40	<0.5	
Etanol + salina (7)	10.34 ± 1.44	<0.001	
Etanol + adenosina (5)	5.37 ± 0.41	<0.005	<0.01

vs. glucosa + salina; ** vs. etanol + salina. Se administraron etanol a dosis de 5 g/Kg o el equivalente calórico de glucosa por vía oral. Los animales se sacrificaron 8 horas después del tratamiento. Los datos se expresan como el promedio ± el error estándar del grupo.

dos que median en la producción de hígado graso por este tóxico, son: abatimiento de la carga energética [28], incremento de la lipólisis en los depósitos debido a la liberación de catecolaminas [41] y alteraciones en la movilización de triglicéridos hepáticos [42]. La tabla IV, presenta los datos obtenidos con tetracloruro de carbono y la reversión parcial de su efecto por la administración de adenosina.

TABLA IV. EFECTO DE LA ADENOSINA SOBRE LA ACUMULACION DE TRIGLICERIDOS PRODUCIDA POR LA ADMINISTRACION DE TETRACLORURO DE CARBONO

Tratamiento	Nivel de Triglicéridos Hepáticos mg/g	p*	p**
Nujol + salina (7)	2.83 ± 0.57		
Nujol + adenosina (7)	3.13 ± 0.27	<0.7	
CCl_4 + salina (7)	17.01 ± 2.60	<0.001	
CCl_4 + adenosina (6)	7.73 ± 1.14	<0.005	<0.01

* vs nujol + salina; ** vs CCl_4 en nujol a dosis de 5 ml/Kg o un volumen equivalente de nujol. Los animales se sacrificaron 6 hs. después del tratamiento. Los datos se expresan como el promedio ± el error estándar del grupo.

Considerando que los hepatotóxicos ensayados actúan en varias formas, no es factible pensar que a su vez la adenosina actúe por un mecanismo diferente antagonizando a cada hepatotóxico; es decir, deben existir mecanismos generales en la producción del hígado graso que son inhibidos por el nucleósido. Existe el problema de que tanto los tóxicos como el nucleósido tienen múltiples efectos y sólo el estudio meticuloso de ellos nos puede conducir a conocer los procesos involucrados y su importancia relativa dentro del contexto general. Nuestro esfuerzo actual está dirigido a la caracterización de estos fenómenos, y esperamos que la integración de las acciones metabólicas de la adenosina, estudiadas por nuestro grupo y otros, nos ayuden en la búsqueda de los mecanismos moleculares comunes a los distintos hepatotóxicos y que puedan ser responsables de la alteración metabólica conocida como hígado graso. Por otra parte abrigamos la esperanza de que la adenosina pueda ser de utilidad práctica para su tratamiento en los humanos.

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On the Mechanism of Ethanol-induced Fatty Liver and Its Reversibility by Adenosine[1]

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The administration of adenosine partially prevented and reverted the ethanol-induced fatty liver. The hepatic α-glycerophosphate concentration and the α-glycerophosphate/dihydroxyacetone phosphate ratio were significantly increased after ethanol administration. The nucleoside decreased with ratio and enhanced the oxidation of ethanol. A strong correlation between the cytoplasmic redox state and the amount of triacylglycerols in the liver was found (8 h after treatments) stressing the paramount importance of the redox state in the pathogenesis of ethanol-induced fatty liver. As previously reported, the nucleoside expanded the adenine nucleotide pool size and the hepatic ATP level. Ethanol potentiated these effects. It is suggested that adenosine ameliorated the ethanol-induced fatty liver through an increased utilization of reducing equivalents by the mitochondria. An interdependence of these effects is proposed and discussed.

It is well known that an acute administration of ethanol causes a reversible fatty liver, characterized mainly by an accumulation of triacylglycerols, both in humans and in experimental animals (1, 2). After the ingestion of ethanol a number of hepatic functions are altered, due partially to the change in the cytosolic redox state ($NAD^+/NADH$) produced by its metabolism. Some reviews treating these processes have appeared (3–5). It has also been reported that a decrease in the hepatic ATP level accompanies the fatty livers produced by ethanol and other hepatotoxins and that, the administration of high doses of ATP protects against acute fatty liver (6). Thus, it has been proposed that ATP depletion is an important factor in the pathogenesis of fatty liver. However, its importance has been questioned by several authors (7, 8).

Previous work has shown that the administration of adenosine produces a marked increase in the hepatic energy charge *in vivo* (9). This finding has been confirmed *in vitro* in perfused rat liver (10), liver slices (11), and isolated hepatocytes (11, 12). Moreover, it has been proposed that adenosine is the most ready precursor for the synthesis of nucleotides in the intact cell through the salvage pathway (12). Nevertheless, the mechanism by which adenosine increases the hepatic energy charge has not been clarified.

The present investigation was undertaken in an effort to get a clearer picture of the pathogenesis of the ethanol-induced fatty liver and of the participation that the depletion of ATP has in it.

MATERIALS AND METHODS

Adenosine, zeolite, glucose-6-phosphate dehydrogenase, lactic dehydrogenase, hexokinase, pyruvate kinase, and myokinase were obtained from the Sigma Chemical Company (St. Louis, Mo.). α-Glycerophosphate dehydrogenase, nucleotides, and coenzymes were purchased from Boehringer and Soehne (Mannheim). The other chemicals used were reagent grade of the best quality available.

The experiments were performed with male Wistar rats weighing between 170 and 210 g which had fasted for 16–20 h. The ethanol given was a 63% (v/v) solution

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in water administered through a stomach tube at doses of 5 g/kg body weight. Control animals received an isocaloric dose of glucose (9.3 g/kg body weight). Immediately after gastric intubation the animals were injected intraperitoneally with saline (0.85% NaCl) or adenosine suspended in saline (20 mg/ml) and adjusted to pH 7.4, at doses of 200 mg/kg body weight. The other conditions used are indicated in the figures and tables.

To quantify the ethanol and lipids in the blood, the animals were decapitated and exsanguinated. Hepatic triacylglycerols were determined by the method of Butler *et al.* (13). Serum triacylglycerols and serum free fatty acids were quantified using the methods of Van Handel and Zilversmit (14) and Dole and Meinertz (15), respectively. Blood ethanol was estimated enzymatically (16). To determine adenine nucleotides, inorganic phosphate, αGP,[2] and DHAP in the liver, the animals were sacrificed by a blow on the head. The abdomen was immediately opened with a bistoury and 150–300 mg of liver were homogenized in 6% (w/v) perchloric acid within 4–7 sec after the abdominal incision. α-Glycerophosphate and DHAP were determined according to Hohorst (16) and Bucher and Hohorst (17), respectively. ATP was quantified using the technique of Lamprecht and Trautschold (18). ADP and AMP were estimated by the method of Adam (19) and incorganic phosphate according to Grindey and Nichol (20).

The energy charge of the adenylate system was calculated according to Atkinson (21) and the phosphorylation potential as described by Stubbs *et al.* (22). The NAD^+/NADH ratio was calculated with the equilibrium constant of the glycerophosphate dehydrogenase obtained by Rüssman (23) and taken from the paper by Veech *et al.* (24). Statistical significance between comparable groups was determined by the Student's test. Linear regressions were calculated by the method of least squares.

RESULTS

The administration of ethanol produced an almost 4-fold increase in the amount of triacylglycerols in the liver 8 h after treatment (Fig. 1). The simultaneous administration of adenosine nearly blocked this effect (Fig. 1). The differences between the groups treated with ethanol + saline and those treated with ethanol plus adenosine were statistically significant at 4 and 8 h ($P < 0.005$ and $P < 0.01$, respectively). Adenosine alone produced only minor modifications in the hepatic triacylglycerol content (Fig. 1).

[2] Abbreviations used: αGP, α-glycerophosphate; DHAP, dihydroxyacetone phosphate.

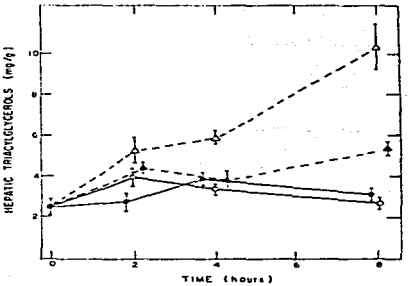

FIG. 1. Time-course of the effect of ethanol and adenosine on the hepatic concentration of triacylglycerols. ○——○, glucose + saline; ●——●, glucose + adenosine; △----△, ethanol + saline; ▲----▲, ethanol + adenosine. Vertical bars represent the standard error of the mean of at least five animals.

The possibility that adenosine might be able not only to prevent the ethanol-induced fatty liver but also to revert it was also tested. Adenosine was injected six hours after the administration of ethanol and the animals were killed two hours later. The nucleoside was found to revert the fatty liver significantly under these conditions (Table I). Subsequent determinations were made 8 h after the simultaneous administration of ethanol or glucose and adenosine or saline except where indicated.

In the ethanol-induced fatty liver most of the fatty acids in the accumulated triacylglycerols derive from adipose tissue (25). It has been proposed that the underlying mechanism is an increased mobilization of free fatty acids from the depots (26). The possibility of adenosine decreasing the mobilization of fatty acids was tested since it is known that the nucleoside is a potent inhibitor of lipolysis in adipose tissue (27, 28) and it has been suggested that it might play a role as a regulator of adenylate cyclase activity (29). The results are presented in Table II where it can be seen that none of the treatments modified to an appreciable extent the concentration of free fatty acids in the plasma.

Another possible explanation for the ac-

TABLE I

PARTIAL REVERSION OF THE ETHANOL-INDUCED FATTY LIVER BY ADENOSINE[a]

Treatment	Hepatic triacylglycerols (mg/g wet weight)
Glucose + saline	3.09 ± 0.43 (9)
Glucose + adenosine	3.34 ± 0.79 (10)
Ethanol + saline	12.85 ± 2.16[b] (11)
Ethanol + adenosine	6.95 ± 1.11[c,d] (10)

[a] Ethanol or glucose was administered 8 h before sacrificing and adenosine or saline 2 h before sacrificing. The results are expressed as the mean ± the standard error of the mean with the number of observations in parentheses.

[b] $P < 0.001$ compared to the glucose + saline group.

[c] $P < 0.005$ compared to the glucose + saline group.

[d] $P < 0.05$ compared to the ethanol + saline group.

TABLE II

EFFECTS OF ETHANOL OR GLUCOSE AND ADENOSINE OR SALINE ON SERUM LIPIDS[a]

Treatment	Free fatty acids (μEq/liter)	Triacylglycerols (mg/100 ml)
Glucose + saline	361.90 ± 41.04 (5)	56.09 ± 6.53 (4)
Glucose + adenosine	444.51 ± 26.23 (4)	59.82 ± 3.74 (4)
Ethanol + saline	368.68 ± 20.50 (5)	101.26 ± 12.85[b] (6)
Ethanol + adenosine	394.68 ± 29.14 (6)	60.96 ± 3.28[c] (5)

[a] The results are expressed as the mean ± the standard error of the mean with the number of observations in parentheses. Determinations were made 8 h after treatment.

[b] $P < 0.02$ compared to the glucose + saline group.

[c] $P < 0.02$ compared to the ethanol + saline group.

tion of the nucleoside on the fatty liver is that adenosine increased the release of triacylglycerols by the hepatocytes. Thus, the amount of serum triacylglycerols was quantified. Ethanol produced a hypertriacylglycerolemia (Table II) as has been previously shown (5). Contrary to what was expected, the administration of adenosine to ethanol-treated rats did not enhance the lipemia but rather decreased it to normal levels (Table II). Adenosine alone had no effect on this parameter (Table II).

Ethanol decreases the cytoplasmic $NAD^+/NADH$ ratio. This effect results from the oxidation of ethanol by NAD^+ acting through alcohol dehydrogenase. In the liver this cytoplasmic enzyme shares a common NAD^+ pool with lactate and αGP dehydrogenases and is in close equilibrium with them (30). As a consequence, ethanol metabolism increases the lactate/pyruvate and αGP/DHAP ratios which reflect the cytoplasmic redox state (24, 30). The time-course action of ethanol and the nucleoside on this parameter was studied and the results are presented in Table III. As expected, the administration of ethanol caused an accumulation of the reduced metabolite and the injection of adenosine partially blocked this effect at all the times tested (Table III). The administration of glucose and adenosine did not modify the cytoplasmic redox state (Table III). Furthermore, strong correlation was observed between the amount of neutral fats and the cytoplasmic redox state in the liver 8 h after treatment, as presented in Fig. 2.

A decrease in ethanol metabolism by adenosine could explain the observed changes in the redox state (Table III) but, as a consequence, the blood levels of ethanol should be higher. It was found that adenosine did not produce any increase in the blood level of ethanol concurrent with the increase in the $NAD^+/NADH$ ratio (Fig. 3). On the contrary, it was observed that the administration of the nucleoside to ethanol-treated rats produced a faster recovery of motor coordination in the animals. The possibility that adenosine might increase ethanol oxidation was considered. It was observed that 12 h after ethanol administration the blood level of ethanol was lower in rats treated with adenosine than in the group treated with saline ($P < 0.005$) and the blood ethanol clearance was about 20% higher in rats treated with the nucleoside (Fig. 3). In addition, when the dose of ethanol was diminished to 2.5 g/kg body weight, the blood ethanol level was lower in rats treated with adenosine 8 h after the administration (5.66 ± 1.11 mM in the ethanol + saline group and 0.90 ± 0.26 mM in the ethanol + adenosine group; mean ± the standard error of the mean of five animals, $P < 0.005$).

TABLE III

EFFECT OF ETHANOL OR GLUCOSE AND ADENOSINE OR SALINE ON THE HEPATIC CYTOPLASMIC REDOX STATE[a]

Time (hours)	Treatment	αGP	DHAP	αGP/DHAP	NAD^+/NADH
		(nmol/g wet weight)			
0	None	184.30 ± 28.74 (5)	34.88 ± 7.36 (5)	5.3	1455.8
2	Glucose + saline	185.85 ± 13.67 (5)	38.66 ± 3.76 (5)	4.8	1599.2
2	Glucose + adenosine	188.43 ± 26.14 (6)	41.47 ± 1.54 (6)	4.5	1694.3
2	Ethanol + saline	664.33 ± 87.22[b] (6)	33.71 ± 3.89 (6)	19.7	390.3
2	Ethanol + adenosine	420.14 ± 38.65[b,c] (5)	40.03 ± 4.76 (5)	10.5	732.6
4	Glucose + saline	185.94 ± 19.08 (6)	33.17 ± 6.80 (6)	5.6	1371.2
4	Glucose + adenosine	206.01 ± 27.26 (5)	30.78 ± 5.83 (5)	6.7	1149.8
4	Ethanol + saline	708.67 ± 41.07[d] (5)	33.66 ± 7.53 (5)	21.1	365.4
4	Ethanol + adenosine	515.26 ± 60.36[d,e] (5)	39.89 ± 3.97 (5)	12.9	595.4
8	Glucose + saline	218.65 ± 25.09 (5)	24.70 ± 4.24 (5)	8.9	869.2
8	Glucose + adenosine	284.05 ± 43.35 (5)	31.91 ± 6.68 (4)	8.9	864.3
8	Ethanol + saline	1163.10 ± 79.45[b] (5)	26.41 ± 5.14 (5)	44.0	174.7
8	Ethanol + adenosine	801.59 ± 42.28[f,g] (5)	36.28 ± 8.86 (5)	22.1	348.2

[a] The results are expressed as the mean ± the standard error of the mean with the number of observations in parentheses.

[b] $P < 0.001$ compared to the glucose + saline group (2 h).

[c] $P < 0.05$ compared to the ethanol + saline group (2 h).

[d] $P < 0.001$ compared to the glucose + saline group (4 h).

[e] $P < 0.05$ compared to the ethanol + saline group (4 h).

[f] $P < 0.001$ compared to the glucose + saline group (8 h).

[g] $P < 0.005$ compared to the ethanol + saline group (8 h).

The hepatic content of adenine nucleotides and inorganic phoshate was studied 2 and 8 h after treatment (Table IV). Two hours after the administration of adenosine the total amount of adenine nucleotides, ATP and the energy charge were increased (Tables IV and V) as previously reported (9) ($P < 0.02$, $P < 0.01$, and $P < 0.02$, respectively). It was also observed that the nucleoside decreased the hepatic content of inorganic phosphate (31) and magnified the phosphorylation potential (Tables IV and V). Ethanol elevated both the level of inorganic phosphate and the level of AMP (Table IV), the simultaneous administration of ethanol and adenosine produced a further enlargement in the adenine nucleotide pool size and notably increased the level of ATP (Table IV and V). However, all these effects were transient because 8 h after treatment no significant differences were observed (Tables IV and V).

DISCUSSION

The ability of adenosine to prevent (Fig. 1) and even to partially revert (Table I) the ethanol-induced fatty liver does not seem to be related to changes in the mobilization of lipids either by adipose tissue or by the liver. The level of serum free fatty acids

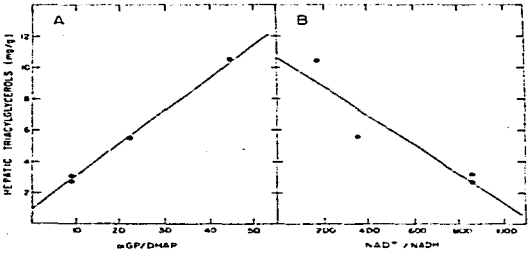

FIG. 2. Correlation between the cytoplasmic redox state and the amount of triacylglycerols in the liver 8 h after treatment. The data were taken from Fig. 1 and Table III. Regression equations and correlation coefficients are: (panel A) $y = 0.21x + 0.94$, $r = 0.99$; (panel B) $y = 0.009x + 10.46$, $r = 0.91$.

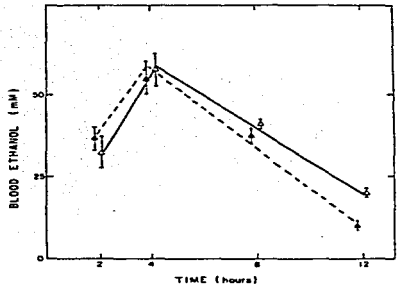

FIG. 3. Blood ethanol concentration in rats treated either with saline or adenosine. Vertical bars represent the standard error of the mean of at least four animals. Regression equations and correlation coefficients were calculated omiting the data for 2 h. △——△ ethanol + saline: $y = 4.71x + 77.28$, $r = 0.94$; ▲----▲ ethanol + adenosine $y = 5.78x + 80.38$, $r = 0.95$.

was not modified by treatment with adenosine (Table II) and the content of serum triacylglycerols was altered by the nucleoside in a direction opposite to that expected from an increase in mobilization (Table II). In the presence of a moderately fatty liver adenosine decreased the amount of triacylglycerols in the serum to normal levels (Table II and Fig. 1). These results suggest that the output of neutral fats by the liver is not a process that follows first order kinetics and that therefore there exists a threshold in the production of alcoholic hyperlipemia. At any rate, in these experiments it is not possible to exclude the interaction between organs. The serum levels of triacylglycerols might not reflect the liver output but a balance between production and utilization. Obviously a more direct approach will be necessary to clarify this point.

Ethanol may profundly influence free fatty acid and triacylglycerol transport. It has been observed that in rats rendered hyperlipemic by ethanol feeding, the lipid/protein ratio of the lipoproteins of density <1.006 approaches that of chylomicrons, but the site of origin of these particles has not been deduced with certainty from physical or chemical characteristics (5, 32). In addition, it has been observed that ethanol causes the liver to receive an increased flow of blood that produces an increase in the hepatic uptake of fatty acids (33).

The most characteristic metabolic alteration during ethanol oxidation is the decrease in the $NAD^+/NADH$ ratio in the liver. In the present work (Table III) this effect was evaluated as an increase in the hepatic αGP/DHAP ratio. Ethanol produced about a 4-fold increase in the αGP/DHAP ratio and in the ethanol + adenosine treated group a decrease in this

TABLE IV

EFFECT OF ETHANOL OR GLUCOSE AND ADENOSINE OR SALINE ON THE HEPATIC LEVELS OF ADENINE NUCLEOTIDES AND INORGANIC PHOSPHATE[a]

Time (hours)	Treatment	ATP	ADP	AMP	P_i
		(μmol/g wet weight)			
2	Glucose + saline	2.99 ± 0.18 (4)	1.29 ± 0.15 (4)	0.26 ± 0.02 (4)	3.42 ± 0.12 (4)
2	Glucose + adenosine	4.29 ± 0.27[b] (4)	1.03 ± 0.08 (4)	0.19 ± 0.05 (4)	2.54 ± 0.10[b] (4)
2	Ethanol + saline	2.84 ± 0.13 (4)	1.45 ± 0.18 (4)	0.43 ± 0.05[c] (4)	4.40 ± 0.16[b] (3)
2	Ethanol + adenosine	5.00 ± 0.08[b,d,e] (4)	1.25 ± 0.16 (4)	0.35 ± 0.04 (4)	2.56 ± 0.05[f,g] (3)
8	Glucose + saline	3.23 ± 0.20 (3)	1.65 ± 0.05 (3)	0.31 ± 0.08 (3)	4.60 ± 0.30 (3)
8	Glucose + adenosine	2.73 ± 0.07 (4)	1.32 ± 0.17 (4)	0.35 ± 0.09 (4)	4.48 ± 0.77 (4)
8	Ethanol + saline	3.32 ± 0.23 (6)	1.40 ± 0.18 (6)	0.26 ± 0.03 (6)	4.02 ± 0.42 (6)
8	Ethanol + adenosine	3.60 ± 0.16 (6)	1.86 ± 0.14 (6)	0.42 ± 0.08 (6)	4.65 ± 0.25 (6)

[a] The results are expressed as the mean ± the standard error of the mean with the number of observations in parentheses.
[b] $P < 0.01$ compared to the glucose + saline group (2 h).
[c] $P < 0.02$ compared to the glucose + saline group (2 h).
[d] $P < 0.05$ compared to the ethanol + saline group (2 h).
[e] $P < 0.05$ compared to the glucose + adenosine group (2 h).
[f] $P < 0.005$ compared to the glucose + saline group (2 h).
[g] $P < 0.001$ compared to the ethanol + saline group (2 h).

TABLE V

EFFECT OF ETHANOL OR GLUCOSE AND ADENOSINE OR SALINE ON ENERGY PARAMETERS OF THE LIVER CELL[a]

Time (hours)	Treatment	Total adenine nucleotides (μmol/g wet weight)	ATP/ADP	Energy charge	Phosphorylation potential (M^{-1})
2	Glucose + saline	4.54	2.32	0.80	678
2	Glucose + adenosine	5.51	4.17	0.87	1640
2	Ethanol + saline	4.72	1.96	0.76	445
2	Ethanol + adenosine	6.60	4.00	0.85	1575
8	Glucose + saline	5.19	1.96	0.78	426
8	Glucose + adenosine	4.40	2.06	0.77	462
8	Ethanol + saline	4.98	2.37	0.81	590
8	Ethanol + adenosine	5.88	1.94	0.77	416

[a] The results were calculated from the data in Table IV.

$$\text{Energy charge} = \frac{1}{2}\left(\frac{[ADP] + 2[ATP]}{[AMP] + [ADP] + [ATP]}\right)$$

$$\text{Phosphorylation potential} = \frac{[ATP]}{[ADP][Pi]}$$

ratio of about 40% was observed at all the times tested. An enhanced metabolism of ethanol (Fig. 3) was observed. Ethanol oxidation is stimulated by substances that increase the electron flux in the mitochondrial respiratory chain and therefore enhance the utilization of reducing equivalents (3, 34–38). It has been concluded that the rate-limiting step in ethanol metabolism is the reoxidation of NADH (3, 38).

Taking into account these observations, the stimulation of ethanol metabolism produced by the nucleoside seems to be a consequence of the modification of the redox state. If adenosine promotes the utilization of reducing equivalents by the mitochondria this would explain the observed increases in ATP, energy charge and phosphorylation potential and the decrease in inorganic phosphate (Tables IV and V). It could also explain the observed increase in the mitochondrial $NAD^+/NADH$ ratio (39) by an enhanced utilization of the reduced form of the coenzyme by the respiratory chain. The magnification by ethanol might result from the avalanche of reducing equivalents that its metabolism causes (Tables III–V). However, the effects on the energy parameters disappeared under conditions in which the action of the redox state was still observable (Tables III, IV and V). Furthermore, Cederbaum *et al.* (38) have suggested that ethanol oxidation does not occur in addition to the oxidation of other substrates and that therefore the reducing equivalents from the oxidation of ethanol may replace substrates normally oxidized by the liver, e.g., fatty acids. It has recently been shown that adenosine inhibits the oxidation of fatty acids (40).

Under the conditions indicated herein, ethanol did not depress hepatic ATP levels (Tables IV and V). In the ethanol + adenosine treated group high levels of ATP were found in rats which were developing a moderately fatty liver (Table IV and Fig. 1). This fact is in agreement with some reports (24, 41, 42) and in disagreement with others (6–8). The discrepancy has been attributed to the dose of ethanol employed, the nutritional condition of the animals and even to the strain of rats used (8). Nevertheless, our results are in agreement with the conclusion that the hepatic ATP depletion does not play a key role in the pathogenesis of the fatty liver produced by ethanol (7, 8).

One point of interest is the strong correlation between the cytoplasmic redox state and the content of hepatic triacylglycerols (Fig. 2). The interpretation of such a correlation is complicated since a decrease in the $NAD^+/NADH$ ratio is mainly accompanied by a simultaneous accumulation of αGP. It has been suggested that αGP is a factor which controls the rate of neutral fat synthesis (43, 44), although its availability is not the sole factor that controls the esterification process (45, 46). In any event, an excess in the oxidation of ethanol promotes the accumulation of αGP by decreasing the $NAD^+/NADH$ ratio. These factors are of paramount importance in the genesis of ethanol-induced fatty liver.

Perhaps two other factors might be involved in the effects of adenosine on fatty liver. This nucleoside may be metabolized to hypoxanthine and afterward to uric acid by xanthine oxidase, generating hydrogen peroxide. Thus, it is possible that catalase participates in an H_2O_2-mediated ethanol peroxidation. This mechanism has the advantage that it does not produce the reduction of coenzymes, which could explain some of the observed effects. However, it is generally accepted that catalase does not play a significant role in ethanol metabolism (for a review see Ref. 47) and it cannot explain the magnification of adenosine action on energy parameters (Tables IV and V).

Evidence that adenosine inhibits extramitochondrial acyl-CoA synthetase has recently been published (40). The activity of this enzyme is essential for the metabolism of fatty acids provided by the depots. However, this effect of adenosine is transient (40). In addition, it has been shown that adenosine inhibits hepatic lipogenesis (11).

In summary, the diminution of the $NAD^+/NADH$ ratio seems to be the main cause of ethanol-induced fatty liver. Adenosine partially prevented or reverted this process (Fig. 1 and Table I) probably by promoting the translocation of reducing power from the cytoplasm to the mitochondria. The molecular mechanisms of these actions are presently under investigation in our laboratory.

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EFFECTS OF ADENOSINE ON ETHANOL-INDUCED MODIFICATIONS OF LIVER METABOLISM

ROLE OF HEPATIC REDOX STATE, PURINE AND FATTY ACID METABOLISM

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Abstract—A total prevention of ethanol-induced fatty liver by the simultaneous administration of adenosine and allopurinol was observed. Under these conditions, adenosine ameliorated the reduction in the cytoplasmic $NAD^+/NADH$ ratio produced by ethanol metabolism, increased the rate of ethanol oxidation, and decreased the blood ketone bodies, reflecting an inhibition of hepatic fatty acid oxidation. Moreover, in rats treated with 4-methylpyrazole, the effect of the nucleoside on the increased rate of ethanol oxidation was not observed. The effect of adenosine on the modifications induced by the administration of ethanol in the mitochondrial redox potential was tested. When the nucleoside was administered to ethanol-treated animals, a reversal of the ethanol-induced diminution in the mitochondrial $NAD^+/NADH$ ratio and in the redox potential was observed after 2–4 hr of treatment. These data lend further support to the suggestion that adenosine promotes the capacity of the cell to handle the reducing equivalents generated during ethanol metabolism. Moreover, these experiments suggest that hydrogen peroxide, generated through purine metabolism, plays a minimal role in the action of adenosine on ethanol metabolism. Finally, a second mechanism by which the nucleoside prevents fatty liver in the presence of allopurinol was evident and this was related to an inhibition of fatty acid metabolism.

It is well known that a great number of hepatic functions are altered after the ingestion of ethanol and that these effects are due mainly to the change in the redox state ($NAD^+/NADH$) produced by its metabolism. In addition, it has been suggested that reoxidation of cytosolic NADH is the rate-limiting step in ethanol metabolism [1–3].

It has also been found that administration of adenosine partially prevents ethanol-induced fatty liver, ameliorates the effect of ethanol metabolism on the cytoplasmic redox state, and increases ethanol oxidation [4]. Furthermore, ethanol enhances the adenosine-mediated increase in ATP [4, 5], and adenosine alone produces a marked shift in the mitochondrial redox state $NAD^+/NADH$ [6]. Taking all these data into account, it has been suggested that the nucleoside may promote translocation of reducing power from the cytoplasm to the mitochondria, and that this mechanism may be responsible for the effects observed [4].

Hepatic cells catabolize adenosine to uric acid very rapidly [7], and in this pathway xanthine oxidase generates hydrogen peroxide. It has been shown that catalase is capable of oxidizing ethanol *in vitro* in the presence of a hydrogen peroxide-generating system [8]. Therefore, although it is generally accepted that, under basal conditions, hydrogen peroxide-mediated ethanol peroxidation does not play a significant role in ethanol metabolism [9, 10], it is theoretically possible that the effects of adenosine on ethanol metabolism might be due to this process.

This point was experimentally tested using allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine], a well-known inhibitor of xanthine oxidase, and 4-methylpyrazole, a potent inhibitor of alcohol dehydrogenase. The present paper shows that the action of adenosine on ethanol metabolism is mediated by alcohol dehydrogenase and the consequent alterations in the cytoplasmic and mitochondrial redox states. It is demonstrated that hydrogen peroxide, generated through purine catabolism, is not an important factor in adenosine action on ethanol-induced metabolic changes. Finally, data are presented showing the effect of the nucleoside on the metabolism of fatty acids originating from triglyceride stores, and the role of these fatty acids in the pathogenesis of ethanol-induced fatty liver.

MATERIALS AND METHODS

Adenosine, allopurinol, zeolite, alcohol dehydrogenase, α-glycerophosphate dehydrogenase, 3-hydroxybutyrate dehydrogenase and uricase were obtained from the Sigma Chemical Co. (St. Louis, MO). Coenzymes were purchased from Boehringer und Soehne (Mannheim, West Germany). 4-Methylpyrazole was a gift from Dr. A. I. Cederbaum. Other chemicals used were reagent grade of the best quality available.

The experiments were performed with male Wistar rats (weighing between 170 and 210 g) which were fasted for 16–20 hr. Ethanol was administered

through a stomach tube at a dose of 2.5 or 5 g/kg body wt. Control animals received an isocaloric dose of glucose. Immediately after gastric intubation, the animals were injected intraperitoneally with saline (NaCl, 0.85%) or adenosine suspended in saline at doses of 200 mg/kg body wt. Allopurinol (20 mg/kg body wt) or 4-methylpyrazole (200 mg/kg body wt) was suspended by homogenization either in saline or in the adenosine suspension. Thus, the animals were injected only once and were not subjected to any extra water or electrolyte load.

Hepatic triacylglycerols were determined by the method of Butler *et al.* [11]. Serum triacylglycerols and serum free fatty acids were assayed according to the methods of Van Handel and Zilversmit [12] and Dole and Meinertz [13], respectively. Blood and hepatic ketone bodies were quantified enzymatically: 3-hydroxybutyrate (3-OHB) according to Williamson and Mellamby [14], and acetoacetate (AA) according to Mellamby and Williamson [15]. Total ketone bodies were calculated as the sum of 3-OHB plus AA. Blood ethanol [16] and plasma uric acid [17] were estimated enzymatically. For the preparation of liver extracts, the animals were killed by a blow on the head. The abdomen was opened immediately with a bistoury and 150–300 mg of liver were homogenized in ice-cold 6% (w/v) perchloric acid within 7 sec after the abdominal incision. The homogenate was centrifuged at 300 *g* for 10 min, and the supernatant fraction was removed and carefully neutralized with 5 M K_2CO_3. Hepatic α-glycerophosphate (α-GP) and dihydroxyacetone phosphate (DHAP) were determined by the methods of Hohorst [18] and Bucher and Hohorst [19], respectively, and 3-hydroxybutyrate and acetoacetate as mentioned previously.

The activity of hepatic xanthine oxidase was quantified in a whole homogenate (20% in 0.25 M sucrose) according to Luck [20]. The activity is expressed as mK/mg protein, where K is the activity number of xanthine oxidase [20]. Protein was determined by the biuret method [21]. The cytoplasmic $NAD^+/NADH$ ratio was calculated using the equilibrium constant of α-glycerophosphate dehydrogenase as obtained by Russman [22] and taken from the paper by Veech *et al.* [23]. The mitochondrial redox state was calculated from the 3-OHB/AA ratio, using the equilibrium constant reported by Krebs [24] for the 3-hydroxybutyrate dehydrogenase. Redox potentials were calculated using the Nernst equation:

$$E_h = E_0' + 0.03 \log \frac{\text{electron acceptor}}{\text{electron donor}}$$

$$E_h = 0.314 + 0.03 \log \frac{NAD^+}{NADH}.$$

Statistical significance between comparable groups was determined by Student's *t*-test.

RESULTS AND DISCUSSION

The ability of allopurinol to inhibit xanthine oxidase was tested. Eight hr after administration, allopurinol produced about 30 per cent inhibition of the activity of this enzyme (Table 1). Surprisingly, adenosine or adenosine + ethanol magnified this effect, a 60 per cent inhibition (compared to the glucose + saline group) being observed in both cases (Table 1). Adenosine alone produced a small inhibition, while only minor changes in the activity of the enzyme were observed with ethanol (Table 1). The additive action of allopurinol and adenosine on xanthine oxidase may be due to a mutual competition for their metabolism. However, the *in vivo* inhibition of purine catabolism in the presence of allopurinol was 95 per cent, since a drop of this magnitude was observed in plasma uric acid levels (results not shown). In any event, the amount of hydrogen peroxide generated through the catabolism of purine was probably diminished to a minimum in rats treated with ethanol + adenosine + allopurinol.

Table 1. Effects of ethanol or glucose and adenosine or saline on the inhibition of xanthine oxidase by allopurinol*

Treatment	Activity of xanthine oxidase (mK/mg protein)	
	Without allopurinol	With allopurinol
Glucose + saline	246.57 ± 12.59 (4)	168.37 ± 26.01† (4)
Glucose + adenosine	203.59 ± 23.37 (3)	102.07 ± 5.39‡§ (4)
Ethanol + saline	229.20 ± 17.94 (4)	152.65 ± 19.28‖ (3)
Ethanol + adenosine	195.86 ± 6.50¶ (3)	99.61 ± 23.29** (4)

* Results are expressed as means ± S.E.M. with the number of animals in parentheses. Determinations were made 8 hr after treatment.

† $P < 0.05$, compared to the glucose + saline group (without allopurinol).

‡ $P < 0.01$, compared to the glucose + adenosine group (without allopurinol).

§ $P < 0.05$, compared to the glucose + saline group (with allopurinol).

‖ $P < 0.05$, compared to the ethanol + saline group (without allopurinol).

¶ $P < 0.02$, compared to the glucose + saline group (without allopurinol).

** $P < 0.02$ compared to the ethanol + adenosine group (without allopurinol).

The ability of adenosine to prevent the ethanol-induced fatty liver was tested in the presence and absence of allopurinol. Eight hr after ethanol administration a 4-fold accumulation of triacylglycerol in the liver was observed, which was partially reversed by adenosine (48 per cent diminution), as reported previously [4]. These effects were essentially unaltered by xanthine oxidase inhibition, but when allopurinol and adenosine were administered together, no ethanol-induced accumulation of lipids was detected. A value of 2.87 ± 0.49 mg hepatic triacylglycerols/g wet wt was detected in the group given glucose and saline, which is similar to that obtained in the rats subjected to ethanol + adenosine treatment (2.15 ± 0.45 mg triacylglycerols/g wet wt) (figures are expressed as the mean ± S.E. of six determinations in each group). Thus, a total prevention of ethanol-induced fatty liver, in the presence of adenosine and allopurinol, was demonstrated.

To study ethanol oxidation, a small dose of ethanol (1 g/kg) was given to rats treated with allopurinol or allopurinol + adenosine, the animals being killed 4 hr after treatment. Under these conditions, the blood level of ethanol was 9.44 ± 1.44 mM in rats treated with allopurinol and 4.92 ± 0.26 mM in rats treated with allopurinol + adenosine (mean ± S.E.M. of five determinations in each case, $P < 0.02$). To further test if there was an increase in ethanol oxidation, 4-methylpyrazole, a specific inhibitor of alcohol dehydrogenase, was employed. Ethanol (2.5 g/kg body weight) was administered to rats treated with pyrazole or pyrazole + adenosine, and the levels of ethanol in the blood were subsequently measured. No significant difference was detected between the groups (results not shown), suggesting that adenosine treatment increased the flux of ethanol *in vivo* through alcohol dehydrogenase.

A clear correlation between liver triacylglycerol content and cytoplasmic redox state was presented in a previous paper [4]. Therefore, the cytoplasmic redox state calculated from the levels of a cytoplasmic pair α-GP and DHAP, was studied in rats treated with allopurinol. The administration of allopurinol did not affect significantly the cytoplasmic redox state in the presence of ethanol with or without adenosine. As expected, ethanol produced a large accumulation of α-glycerophosphate, reflecting a drastic decrease in the cytoplasmic $NAD^+/NADH$ ratio. Adenosine significantly ameliorated this effect of ethanol metabolism by a magnitude similar to that observed previously in the absence of allopurinol [4]. The strong correlation previously observed between the cytoplasmic redox state and the amount of triacylglycerols in the liver [4] was not observed in rats treated with allopurinol, the correlation coefficient being 0.56. This is probably due to the fact that adenosine completely prevented the accumulation of triacylglycerols produced by ethanol and partially modified the effect of the alcohol on the redox state, increasing by 30–40 per cent the $NAD^+/NADH$ over the control group. Hence, in addition to its effect on the cytoplasmic redox state, adenosine probably prevents the ethanol-induced fatty liver by a second mechanism, as evidenced in the presence of allopurinol.

In an effort to clarify this second mechanism manifested by adenosine, the concentration of serum lipids and the blood level of ketone bodies, as an index of the oxidation of fatty acids by the liver [25], were investigated. Allopurinol did not modify to an appreciable extent the effect of adenosine alone, or in the presence of ethanol, on the level of triacylglycerols in serum. As reported previously, ethanol produced an increase in serum triacylglycerols, and the administration of adenosine did not increase the triacylglycerolemia but decreased it to normal levels. It is interesting that in the four experimental groups allopurinol treatment decreased triacylglycerolemia by about 20 per cent.

Adenosine is a powerful antilipolytic agent [26] and, although this action does not seem to play an important role in its effect on fatty liver [4], the possibility of a magnification by allopurinol of the antilipolytic action of adenosine was considered. Contrary to what was expected, in the presence of allopurinol, which inhibits purine catabolism, adenosine increased serum-free fatty acid levels, the concentrations detected being 41.5 ± 7.6 μ-equiv./100 ml of serum in the glucose + saline group and 56.6 ± 4.7 μ-equiv./100 ml in the presence of adenosine. This action was more evident in animals treated with ethanol, where a marked increase in serum-free fatty acids was observed (54.5 ± 2.3 in the ethanol + saline group and 68.8 ± 43.3 in the ethanol + adenosine-treated animals; mean ± S.E. of samples taken from six animals in each experimental group after 8 hr of the treatment, $P < 0.02$).

The level of free fatty acids in the serum of rats is the result of a balance between lipolysis, mainly in adipose tissue, and utilization, mainly by the liver. Savolainen *et al.* [27] have shown that *in vivo* ethanol administration increases adipose tissue cyclic AMP. However, no significant increases in serum-free fatty acids were observed by these authors [27] or by us. This is probably due to the increased hepatic uptake of free acids from serum in ethanol-treated rats [28]. The increased serum level of fatty acids observed in rats treated with ethanol + adenosine + allopurinol probably resulted from an enhanced lipolysis produced by ethanol and a decreased free fatty acid uptake by the liver due to adenosine [29]. On the other hand, the action of adenosine in inhibiting hepatic fatty-acid oxidation that results in decreased plasma ketone body levels [28] was observed in the presence of allopurinol. This action of the nucleoside was more evident after 4 hr of treatment, as evidenced by an inhibition of the ketonemia of 77 per cent in the animals receiving a glucose load and 54 per cent in those treated with ethanol. Ethanol increased the amount of 3-OHB and the total amount of ketone bodies, and both actions were blocked by adenosine. The results previously obtained [30] in the absence of allopurinol persisted for 1 hr, but in the presence of this drug the effect was optimal after 4 hr of treatment, and a 20 per cent inhibition of ketonemia was still evident after 8 hr. As yet, we have no explanation for the observed effects of allopurinol in prolonging the action of adenosine. However, under these conditions, fatty acids were neither esterified nor oxidized, i.e. no accumulation of triacylglycerols in the liver was detected, the

hyperlipemia produced by ethanol was not increased but rather decreased to normal levels, and the concentration of ketone bodies was not augmented but decreased. Therefore, a reasonable explanation of these observations is that these fatty acids are not metabolized by the liver under these conditions. This point is supported by the fact that adenosine inhibits the extramitochondrial activation of fatty acids [30] which is essential for their hepatic metabolism.

The effects of adenosine alone on the mitochondrial redox state have already been published and discussed [6] and are consistent with the hypothesis that the nucleoside enhances the utilization of reducing power by the respiratory chain. The results obtained for these variables in the presence of ethanol and/or adenosine and their controls, receiving a glucose load, are presented in Table 2. One hour after treatment with glucose the animals exhibited a low rate of fatty acid oxidation. If ketogenesis is considered an index of hepatic fatty acid oxidation (Table 2), this probably occurs because, following glucose administration, the liver should have a low metabolic rate, since glucose is available for consumption by extrahepatic tissues, and it is not necessary for the liver to produce ketone bodies or glucose. However, the mitochondrial redox state, expressed as $NAD^+/NADH$, was kept at the normal value reported previously [6]. After 4 hr of treatment, a decrease in the mitochondrial $NAD^+/NADH$ ratio in the control animals receiving a glucose load was observed, probably due to hormonal adjustment, since the glucose absorption must be terminated after the second hour. Adenosine action was observed in these conditions, and 1 hr after the treatment of glucose and adenosine a marked decrease in 3-OHB (reduced substrate) was observed. Consequently, the $NAD^+/NADH$ ratio increased, which is in agreement with data reported previously without the glucose load [6].

The metabolism of ethanol produces acetaldehyde which is oxidized within the mitochondria by acetaldehyde dehydrogenase, generating NADH and, so, exceeding the capacity of utilization by the respiratory chain thereby modifying the mitochondrial redox state. As expected, ethanol produced a marked decrease in the mitochondrial $NAD^+/NADH$ ratio at all times tested, due mainly to an appreciable increase of 3-OHB (reduced substrate).

The administration of adenosine produced an increase in the metabolism of ethanol [4], thus increasing the supply of acetaldehyde to the mitochondria and, consequently, leading to a further excess of NADH which will result in a decrease of the $NAD^+/NADH$ ratio more marked than with ethanol alone. The action of adenosine, however, was exactly the opposite, i.e. the nucleoside increased the mitochondrial redox state (Table 2) by decreasing the level of 3-OHB (reduced substrate) until 4 hr of the treatment. No significant changes in the levels of acetoacetate were observed after nucleoside treatment. The action of adenosine on these variables was not evident after 8 hr of treatment. These data lend further support to the suggestion that reducing equivalents generated by the oxidation of ethanol are utilized faster by the respiratory chain. This could be a consequence of an increased transfer of reducing power from the cyto-

Table 2. Effects of ethanol or glucose and adenosine or saline on the hepatic mitochondrial redox state

Time (hr)	Treatment	3-OHB (μmoles/g wet wt)	AA (μmoles/g wet wt)	Total ketone bodies (3-OHB + AA) (μmoles/g wet wt)	$NAD^+/NADH$
1	Glucose + saline	163.7 ± 21.3	196.4 ± 36.8	360.2	24.44
1	Glucose + adenosine	48.8 ± 7.01	196.6 ± 27.5	245.4	81.63
1	Ethanol + saline	585.7 ± 87.7†	256.6 ± 38.9	842.3	8.90
1	Ethanol + adenosine	501.1 ± 59.8†	229.6 ± 44.1	730.7	9.29
2	Glucose + saline	136.7 ± 18.7	149.6 ± 43.5	286.4	22.29
2	Glucose + adenosine	129.7 ± 22.5	203.3 ± 27.4	333.0	31.69
2	Ethanol + saline	531.3 ± 50.9‡	175.90 ± 43.53	707.2	6.72
2	Ethanol + adenosine	351.9 ± 29.2§‖	171.6 ± 32.3	523.5	9.89
4	Glucose + saline	346.0 ± 44.5	211.7 ± 25.5	557.7	12.44
4	Glucose + adenosine	198.0 ± 30.1¶	225.6 ± 46.9	423.6	23.05
4	Ethanol + saline	886.2 ± 113.7**	325.8 ± 46.3	1212.0	7.46
4	Ethanol + adenosine	599.0 ± 100.7††	325.5 ± 53.2	924.5	11.02
8	Glucose + saline	291.2 ± 24.1	240.1 ± 42.4	531.3	16.76
8	Glucose + adenosine	288.1 ± 40.9	233.3 ± 34.6	521.3	16.49
8	Ethanol + saline	568.5 ± 101.8‡‡	223.0 ± 38.0	791.4	7.95
8	Ethanol + adenosine	671.1 ± 117.52§§	211.17 ± 23.82	882.3	6.38

* Results are expressed as the mean ± S.E.M. of at least four animals for each group.
† $P < 0.005$, compared to the glucose + saline group (1 hr).
‡ $P < 0.001$, compared to the glucose + saline group (2 hr).
§ $P < 0.001$, compared to the glucose + saline group (2 hr).
‖ $P < 0.02$, compared to the ethanol + saline group (2 hr).
¶ $P < 0.025$, compared to the glucose + saline group (4 hr).
** $P < 0.005$, compared to the glucose + saline group (4 hr).
†† $P < 0.05$, compared to the glucose + saline group (4 hr).
‡‡ $P < 0.025$, compared to the glucose + saline group (8 hr).
§§ $P < 0.01$, compared to the glucose + saline group (8 hr).

plasm to the mitochondria, an increase in the flux of the respiratory chain, or by a synergy of the two mechanisms. Although this point will not be discussed here, in Table 3 we present the mitochondrial redox potential calculated from Table 2, the cytoplasmic redox potentials calculated from data in our previous paper [4], and the differences between both compartments. Adenosine alone produced an increase of 15 mV in the mitochondrial redox potential 1 hr after its administration. This action diminished to 4 and 8 mV after 2 and 4 hr, respectively, and then disappeared. No effect of adenosine alone on the cytoplasmic redox potential was detected (Table 3). Ethanol decreased (became more negative) the redox potential in both compartments. In relation to its effects on ethanol metabolism, the nucleoside produced an elevation of 5 mV in the mitochondrial compartment and 5–6 mV in the cytoplasm after 1, 2 and 4 hr of the treatment. Eight hr after treatment the action of adenosine on the cytoplasm was still apparent, although no effect was detected within the mitochondria. It is interesting, however, that adenosine only modifies the cytoplasmic redox state when there is a low $NAD^+/NADH$ ratio, i.e. during ethanol oxidation. The differences in redox potential between the two compartments remained fairly constant during the course of the experiment (Table 3), regardless of the treatment and of the cytoplasmic and mitochondrial modifications induced by ethanol alone or with adenosine. Although at the moment it is difficult to understand the physiological meaning of the requirement to keep at around 50 mV the difference between cytoplasmic and mitochondrial redox potential, it is evident that the constancy of this equilibrium was maintained. No evidence for a direct interaction of adenosine and ethanol metabolism is presented, but it seems to involve an indirect action mediated through fatty acid and mitochondrial metabolisms. The inhibition of fatty acid oxidation by ethanol has been reported [31], the effect being measurable after 1 or 2 hr of the treatment (Table 2) and potentiated by the presence of adenosine. It is important to emphasize that the inhibition of fatty acid oxidation induced by ethanol is accompanied by an increase in triglyceride biosynthesis, whereas adenosine inhibition is concomitant with an inhibition in triglyceride formation.

In summary, two factors seem to be involved in the pathogenesis of ethanol-induced fatty liver: an increased supply of fatty acids to the liver concomitant with an enhanced uptake and an increased availability of α-GP. Adenosine action on ethanol-induced fatty liver does not appear to be related to an H_2O_2-mediated ethanol peroxidation but to its effect on the cytoplasmic redox state limiting the availability of α-GP, and to an enhanced translocation and oxidation of the reducing power.

In the presence of allopurinol, another mechanism of adenosine-mediated prevention of ethanol-induced fatty liver became evident, an inhibition of fatty acid metabolism. Therefore, adenosine prevents fatty liver by limiting the availability of the precursors for triacylglycerol synthesis, α-GP and acylCoA. At the moment, however, it is difficult to correlate both actions of adenosine that prevent the ethanol-induced fatty liver.

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Table 3. Comparative action of ethanol or glucose and adenosine or saline on the cytoplasmic and mitochondrial redox potentials*

Time (hr)	Treatment	$E_{Cytoplasmic}$ (mV)	$E_{Mitochondrial}$ (mV)	$E_{(C-M)}$ (mV)
1	Glucose + saline		−272.36	
1	Glucose + adenosine		−256.64	
1	Ethanol + saline		−285.52	
1	Ethanol + adenosine		−284.96	
2	Glucose + saline	−217.88	−273.56	55.68
2	Glucose + adenosine	−217.13	−268.97	51.84
2	Ethanol + saline	−236.26	−289.18	52.92
2	Ethanol + adenosine	−228.05	−284.14	56.09
4	Glucose + saline	−219.89	−281.16	61.27
4	Glucose + adenosine	−222.18	−273.12	50.94
4	Ethanol + saline	−237.12	−287.82	50.70
4	Ethanol + adenosine	−230.76	−282.73	51.97
8	Glucose + saline	−225.84	−277.27	51.43
8	Glucose + adenosine	−225.90	−277.48	51.58
8	Ethanol + saline	−246.73	−286.99	40.26
8	Ethanol + adenosine	−237.74	−289.86	52.12

* Results were calculated from data taken from Table 1 and Ref. 1.

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MECHANISM OF THE FATTY LIVER INDUCED BY CYCLOHEXIMIDE AND ITS REVERSIBILITY BY ADENOSINE

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Abstract—The administration of cycloheximide to fasted rats produced a 2- to 3-fold increase in hepatic triacylglycerols. The antibiotic decreased the serum level of free fatty acids, triacylglycerols and ketone bodies. It also increased the incorporation of radioactive palmitate into liver lipids. It is suggested that an increased uptake of free fatty acids and an altered partition between oxidation and esterification are involved in the formation of fatty liver by this hepatotoxin. The simultaneous administration of adenosine prevented the formation of fatty liver. It is proposed that the effect of the nucleoside may be related to a decrease in the uptake of fatty acids by the liver.

In recent years there has been a great interest in using inhibitors of protein synthesis to investigate the pathogenesis of the fatty liver. The impairment of the biosynthesis of apolipoproteins and hence of their release has been considered the main factor in the formation of the fatty liver [1–3]. Jazcilevich and Villa-Treviño [4] have shown that the administration of cycloheximide (CHM), a powerful inhibitor of protein synthesis, to rats produces a 2- to 3-fold increase in the amount of triacylglycerols in the liver. This effect of the antibiotic has been confirmed both *in vivo* [5, 6] and *in vitro* [7]. However, Sabesin [8] has shown recently that acetoxycycloheximide, the most powerful inhibitor of protein synthesis in mammalian tissue, neither increased hepatic triacylglycerols nor diminished their concentration in plasma. This author concluded that hepatic inhibition of protein synthesis is not sufficient to produce an accumulation of triacylglycerols in the liver and suggested that in the pathogenesis of the fatty liver produced by CHM an increased mobilization of free fatty acids (FFA) may be involved [8]. Nevertheless, it has been shown previously that CHM does not increase the level of FFA in serum, but rather decreases it [5, 9]. This effect is due to an enhanced esterification of FFA in adipose tissue [9–11].

The present work was undertaken in an effort to get a clearer picture of the mechanism(s) involved in the genesis of the fatty liver produced by CHM. In addition, since it was shown previously that the administration of adenosine partially prevented the ethanol-induced fatty liver [12], the possibility that the nucleoside may protect against the accumulation of lipids in the liver produced by CHM was also tested.

MATERIAL AND METHODS

Cycloheximide, adenosine, 3-hydroxybutyrate dehydrogenase and zeolite were obtained from the Sigma Chemical Company (St. Louis, MO). Palmitic acid[1-^{14}C] as the sodium salt (17.8 mCi/m-mole) and DL-leucine[4,5-^{3}H], (50 mCi/m-mole) were purchased from the International Chemical & Nuclear Corp. (Irvine, CA). Coenzymes were obtained from Boehringer und Soehne (Mannheim). Other chemicals used were reagent grade of the best quality available.

The experiments were performed with male Wistar rats weighing between 140 and 180 g and fasted for 16–20 hr. The animals were injected intraperitoneally with saline (0.85% NaCl) or CHM (3.3 mg/kg of body weight) dissolved in saline and with saline or adenosine (200 mg/kg of body weight) suspended in saline. Therefore, each animal was injected twice, one injection immediately after the other. Four groups of animals were formed in each experiment, i.e. saline + saline, saline + adenosine, CHM + saline and CHM + adenosine. Other conditions used are indicated in the tables or in the text.

Hepatic triacylglycerols were determined by the method of Butler *et al.* [13]. Serum triacylglycerols and serum FFA were quantified using the methods of Van Handel and Zilversmit [14] and Dole and Meinertz [15] respectively. Blood ketone bodies were quantified enzymatically: 3-hydroxybutyrate (3-OHB) and acetoacetate (AA) according to Mellanby and Williamson [16] and Williamson and Mellanby [17] respectively. Total ketone bodies were calculated as the sum of 3-OHB plus AA.

To study hepatic protein synthesis, animals were injected with radioactive leucine (50 μCi/kg body weight) 30 min before being killed. The incorporation of the amino acid into liver proteins and the acid-soluble radioactivity were determined essentially as described by Jazcilevich and Villa-Treviño [4]. The incorporation of radioactive palmitate into liver and adipose tissue lipids was studied in pulse-type experiments in which the animals were injected intraperitoneally with palmitate (10 μCi/kg of body weight). After 15 min they were killed and samples of the liver and the epididymal fat pads were removed, weighed and homogenized. Lipids were extracted by the method of

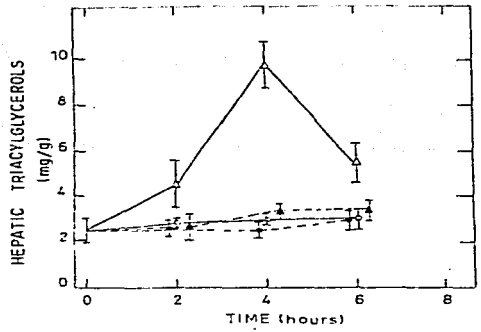

Fig. 1. Time course of the effect of cycloheximide and adenosine on hepatic triacylglycerols. Vertical lines represent the standard error of the mean of at least five determinations. Key: (○——○) saline + saline. (● – – – ●) saline + adenosine. (△——△) cycloheximide + saline, and (▲ – – – ▲) cycloheximide + adenosine.

Folch *et al.* [18] and radioactivity in the lipid extracts was counted as described previously [9]. Statistical significance between comparable groups was determined by Student's *t*-test.

RESULTS

The administration of cycloheximide to fasted male rats increased the amount of triacylglycerols in the liver (Fig. 1). A maximal, 3-fold increase was detected 4 hr after treatment, following which the effect declined (Fig. 1). Adenosine alone produced only minor modifications in the amount of triacylglycerols in the liver but blocked completely the production of fatty liver by CHM (Fig. 1).

Table 1. Effect of cycloheximide and adenosine on the concentration of liver triacylglycerols in male and female rats*

Treatment	Liver triacylglycerols (mg/g)	
	Males	Females
Saline + saline	2.99 ± 0.27 (5)	8.60 ± 1.30 (6)
Saline + adenosine	2.28 ± 0.38 (5)	5.12 ± 1.27 (7)
CHM + saline	9.74 ± 1.04† (5)	22.12 ± 1.91‡ (6)
CHM + adenosine	3.30 ± 0.41§ (5)	14.08 ± 1.08‖ (8)

* Determinations were made 4 hr after treatment. The results are expressed as the mean ± the standard error of the mean with the number of animals in parentheses.
† $P < 0.001$, compared to the saline + saline group (males).
‡ $P < 0.001$, compared to the saline + saline group (females).
§ $P < 0.001$, compared to the CHM + saline group (males).
‖ $P < 0.005$, compared to the CHM + saline group (females).

It is well known that female rats show increased susceptibility to fatty liver induction after treatment with hepatotoxins [19], so the effect of CHM in female rats was tested. It was observed that female rats had an increased amount of triacylglycerols in the liver, compared with males (Table 1). Cycloheximide produced a 2.5-fold increase in the amount of hepatic triacylglycerols in female rats and this effect was also blocked by adenosine (Table 1). In other words, the effects of CHM and adenosine on hepatic triacylglycerols were observed in both male and female rats.

The possibility that adenosine might reverse the inhibition of protein synthesis produced by CHM was considered and tested. As expected, CHM decreased the incorporation of leucine (≈95 per cent) into liver proteins and increased the amount of the label in the acid-soluble fraction (Table 2). Adenosine was unable to reverse this inhibition of protein synthesis and actually seemed to magnify it (Table 2).

The effects of CHM and adenosine on serum lipids were studied. Cycloheximide decreased the amount of triacylglycerols in the serum by 55 per cent and adenosine was unable to restore it to its normal value (Table 3). As has been reported previously, CHM produced a marked decrease (about 40 per cent) in the concentration of FFA in the serum (Table 3) [5, 9] which was not modified appreciably by the nucleoside.

The level of FFA in the serum results from a balance between lipolysis, mainly in adipose tissue, and utilization, mainly in adipose tissue and liver. The incorporation of radioactive palmitate into total lipids of adipose tissue and liver was studied in pulse-type experiments, as an index of the utilization of FFA. Cycloheximide produced marked increases in the incorporation of palmitate in both adipose tissue and liver (Table 4). Adenosine markedly decreased this effect of CHM on the liver but only slightly affected its action on adipose tissue, suggesting a different sensibility (Table 4).

Table 2. Effects of cycloheximide and adenosine on the incorporation of leucine into rat liver protein*

Treatment	Protein (cpm/mg protein)	Acid-soluble fraction (cpm/mg liver)
Saline + saline	327.04 ± 14.83 (5)	8.97 ± 0.28 (5)
Saline + adenosine	316.08 ± 10.76 (5)	10.41 ± 0.79 (5)
CHM + saline	39.42 ± 3.91† (5)	15.92 ± 0.28† (5)
CHM + adenosine	18.64 ± 1.40†‡ (5)	15.92 ± 2.28§ (5)

* Indications as in Table 1.
† P < 0.001, compared to the saline + saline group.
‡ P < 0.005, compared to the CHM + saline group.
§ P < 0.02, compared to the saline + saline group.

The amount of ketone bodies in the blood reflects the oxidation of long-chain fatty acids by the liver [20]. Cycloheximide produced a strong decrease in total ketone bodies that resulted from a diminution in the levels of both 3-OHB and AA without modifying the 3-OHB/AA ratio (Table 5). Adenosine was not able to reverse this effect and no direct action of the nucleoside on this parameter was detected at the time tested (Table 5).

DISCUSSION

The present study confirms that the administration of CHM to rats produces fatty liver (Fig. 1, Table 1) [4–6]. As expected, the antibiotic markedly decreased the serum level of triacylglycerols (Table 3). It also decreased the serum level of FFA (Table 3), as has been shown previously [5, 9]. Hence, the hypothesis that an oversupply of FFA is involved in the pathogenesis of the fatty liver induced by CHM [8] may be discarded.

Table 3. Effects of cycloheximide and adenosine on serum lipids*

Treatment	FFA (μequiv/liter)	Triacylglycerols (mg/100 ml)
Saline + saline	480.78 ± 64.54 (8)	63.12 ± 8.96 (8)
Saline + adenosine	487.33 ± 49.76 (6)	65.94 ± 5.31 (4)
CHM + saline	304.38 ± 39.92† (6)	28.81 ± 3.15‡ (5)
CHM + adenosine	267.82 ± 33.16§ (6)	35.87 ± 4.74† (4)

* Indications as in Table 1.
† P < 0.05, compared to the saline + saline group.
‡ P < 0.01, compared to the saline + saline group.
§ P < 0.02, compared to the saline + saline group.

Table 4. Effects of cycloheximide and adenosine on the incorporation of palmitate into lipids of the liver and epididymal fat pads*

Treatment	Incorporation (cpm/mg wet wt)	
	Liver	Fat pads
Saline + saline	975.16 ± 168.62 (5)	1372.22 ± 227.48 (5)
Saline + adenosine	872.80 ± 170.96 (4)	2382.46 ± 532.12 (4)
CHM + saline	1901.20 ± 79.27† (4)	4570.05 ± 673.25† (4)
CHM + adenosine	920.50 ± 97.08‡ (4)	3269.60 ± 347.99† (4)

* Indications as in Table 1.
† P < 0.005, compared to the saline + saline group.
‡ P < 0.001, compared to the CHM + saline group.

Table 5. Effects of cycloheximide and adenosine on the blood level of ketone bodies*

Treatment	3-OHB	AA	Total ketone Bodies	3-OHB/AA
	(μmoles/100 ml)			
Saline + saline	18.18 ± 1.70 (6)	21.00 ± 2.50 (6)	39.19 ± 2.96 (6)	0.87
Saline + adenosine	20.43 ± 3.05 (6)	21.86 ± 1.86 (6)	42.24 ± 2.89 (6)	0.93
CHM + saline	7.23 ± 1.49† (5)	7.53 ± 1.03† (5)	15.23 ± 2.02† (5)	0.96
CHM + adenosine	9.30 ± 1.30‡ (5)	9.14 ± 1.55‡ (5)	18.44 ± 1.86† (5)	1.02

* Indications as in Table 1.
† $P < 0.001$, compared to the saline + saline group.
‡ $P < 0.005$, compared to the saline + saline group.

The incorporation of radioactive palmitate was increased 2-fold in rats treated with CHM (Table 4). But, since the amount of FFA in the serum of these rats was decreased by 40 per cent (Table 3), the increased incorporation seems to be due to a minor isotopic dilution and therefore the net incorporation of FFA may have been the same. Nevertheless, it is well known that the uptake of FFA by the liver is directly proportional to the concentration to which it is exposed [21]. Since in rats treated with the antibiotic the serum level of FFA was lower, the results suggest that the uptake of FFA by the liver is increased probably due to the structural changes in cellular membranes of the hepatocyte, as reported previously [22]. However, the possibility that the increased incorporation of radioactive palmitate may have resulted from an accumulation of label due to the inhibition of protein synthesis cannot be ruled out completely in spite of the fact that the experiments were pulse-type. The increased incorporation of palmitate into epididymal fat pad lipids is in agreement with the stimulation of the esterification process produced by the antibiotic [9–11].

Cycloheximide decreased the blood level of ketone bodies by approximately 60 per cent (Table 5). This effect may be due to a diminished availability of fatty acids within the hepatocyte, which seems unlikely taking into account the points previously discussed, or to a change in the partition between esterification and oxidation. In addition, preliminary evidence suggests that CHM inhibits the oxidation of FFA. Liver homogenates from CHM-treated rats exhibit a diminished production of AA from exogenously supplied fatty acids.

In conclusion, the production of fatty liver by CHM seems to be due to an enhanced uptake of FFA (Tables 3 and 4) and an altered partition between oxidation and esterification (Tables 1 and 5) in the presence of a strong inhibition of protein synthesis (Table 2).

It was observed that female rats had a larger amount of triacylglycerols in the liver than males (Table 1). This difference has been observed previously by other authors [23] and it is probably related to the different metabolism of free fatty acids by male and female rat livers [24].

Adenosine completely prevented the formation of fatty liver by CHM (Fig. 1 and Table 1). The nucleoside affected neither the inhibition of protein synthesis nor the decrease in serum triacylglycerols produced by the antibiotic (Tables 2 and 3). Therefore, an increased output of fat from the liver is not the mechanism of adenosine action. The effect of CHM to decrease the serum level of FFA was not modified by the nucleoside, but the incorporation of radioactive palmitate into liver lipids was strongly diminished as compared with that of the CHM + saline group (Table 4). This result suggests that livers treated with CHM plus adenosine do not present the enhanced uptake of fatty acids produced by the antibiotic. This point is supported by the inhibition of acyl-CoA synthetase produced by adenosine both *in vivo* and *in vitro* [25]. The activity of this enzyme is essential for the hepatic metabolism of fatty acids supplied by the depots. The effect of adenosine on this enzyme is transient [25], and no action on FFA uptake was observed in rats treated with saline + adenosine (Tables 3 and 4). Therefore, in rats treated with CHM + adenosine, a lengthening of this action of adenosine seems to exist (Tables 3 and 4).

The diminution in the level of serum ketone bodies in rats treated with CHM + adenosine was similar to that observed in rats treated with CHM + saline. It is not clear yet if this action is due to a diminished uptake of FFA (Tables 3 and 4) or to the effect of CHM on FFA oxidation. Previously, it was reported that adenosine decreased the level of ketone bodies in serum [25], but the result observed in rats treated with CHM + adenosine (Table 5) does not seem to be related to this effect of the nucleoside, since adenosine profoundly affects the 3-OHB/AA ratio and in the present study no modification of this parameter was observed.

In a previous paper it was shown that adenosine partially blocked the ethanol-induced fatty liver [12]. This action was strongly related to the effects of ethanol and adenosine on the cytoplasmic redox state [12]. In this paper another mechanism by which adenosine prevents fatty liver is presented: a diminution of the hepatic uptake of fatty acids provided by the depots. Since the enzyme that regulate fatty acid metabolism are membrane associated and CHM has been shown to have an important action in the structure and function of cell membranes of the liver [22], the interaction of the antibiotic with the nucleoside in the induction of the fatty liver could possibly occur at membrane level.

Experiments are in progress to clarify some of the points raised in this discussion.

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"EFFECT OF ACUTE ADMINISTRATION OF ETHANOL AND ADENOSINE ON MITOCHONDRIAL FUNCTION"

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Running title: Adenosine and Mitochondrial Function

SUMMARY

Mitochondria from rats treated with ethanol or adenosine exhibited increased rates of State 3 respiration and phosphorylation rates in the presence of Site I substrates. The effects of ethanol and adenosine were additive. No effect was observed on the oxidation of succinate. Mitochondrial ATPase activity and that of the adenine nucleotide translocator were unaffected by any of the treatments. In order to determine if the action was on the respiratory chain the oxidation of NADH or succinate were studied in sonicated mitochondria and submitochondrial particles. The oxidation of NADH was stimulated by adenosine treatment in both preparations. The data indicate that the administration of adenosine enhances the activity of the mitochondrial respiratory chain and that the effect is persistent after mitochondrial isolation and even after the disruption of its integrity.

1. INTRODUCTION

Adenosine administration to fasted rats inhibits the induction of fatty liver by ethanol and enhances ethanol oxidation (1). Ethanol is mainly oxidized via alcohol dehydrogenase in the cytoplasm generating acetaldehyde and NADH. Acetaldehyde is oxidized in the mitochondria by acetaldehyde dehydrogenase generating acetate and NADH. Therefore an increase in the oxidation of ethanol will increase the supply of reducing power (NADH) to both compartments (cytosol and mitochondria). However, adenosine increases ethanol oxidation but ameliorates the redox state produced by ethanol oxidation in both compartments (1,2). It is generally accepted that the rate limiting step in the metabolism of ethanol is the reoxidation of NADH (3,5) and that ethanol oxidation is stimulated by agents that increase the electron flux through the mitochondrial respiratory chain and therefore enhance the utilization of reducing power (3). Taking into account these data it was suggested that the action of adenosine may be due to an enhanced translocation of reducing power from the cytoplasm to the mitochondria and its utilization by the respiratory chain (1,2). Consistent with this suggestion are the following facts: adenosine administration increase the hepatic level of ATP (1,6), decrease the level of inorganic phosphate and elevates the energy parameters of the hepatocyte (ATP/ADP ratio, phosphorylation potential and energy charge (1,6,7). Furthermore, adenosine markedly decreases the amount of NADH in the mitochondria as reflected by a 7-fold increase in the mitochondrial NAD/NADH ratio (8). The data strongly suggest an enhanced transduction of redox power into high energy bonds. In an effort to gain more insight into the mechanism(s) of action of the nucleoside, the effects of acute administration of ethanol and adenosine on

the functions of hepatic mitochondria were studied.

2. MATERIALS AND METHODS

Adenosine, adenosine-5-diphosphate, adenosine-5-triphosphate, 2,4 dinitrophenol (DNP), dl-alphaglycerophosphate, hexokinase, glucose-6-phosphate dehydrogenase, glutamic acid and succinic acid were obtained from the Sigma Chemical Company (St. Louis, Mo.,). Nicotinamide adenine dinucleotide in the reduced form (NADH) was obtained from Boehringer und Sohne (Mannheim). [8-^{14}C] Adenosine-5-diphosphate (51mCi/mmole) was purchased to Amersham (Arlington Height, IL). Other chemicals used were reagent grade of the best quality available.

The experiments were performed with male Wistar rats, weighing between 170 and 210 g, which were fasted for 16-20 hours. Ethanol was administered through a stomach tube at a dose of 5 g/Kg body weight. Control animals received an isocaloric dose of glucose (9.3 g/Kg body weight). Immediately after gastric intubation, the animals were injected intraperitoneally with saline (0.85% NaCl) or adenosine (200 mg/Kg body weight) suspended in saline. The animals were sacrificed 2 hours after treatment by decapitation and exsanguinated. Liver mitochondria were isolated by differential centrifugation according to the procedure of Schneider and Hogeboon (9), and stored in ice at a concentration of 40 mg protein/ml. Sonicated mitochondria were prepared as follows: isolated mitochondria suspended in 5 ml of 0.25 M sucrose, 1 mM EDTA, 1 mM tris/HCl pH 7.3 (20 mg protein/ml) were sonicated in a type 7685/2 Mullar apparatus for 3 periods of 2 minutes. After this treatment marked translucency changes were observed. The suspension was centrifuged at 15,000 x g for 10 min to eliminate intact mitochondria (pellet) and the supernatant saved (sonicated mitochondria). Submitochondrial particles were prepared as

follows: sonicated mitochondria (15,000 x g supernatant) were centrifuged at 100,000 x g for 40 min, the supernatant was discared and the pellet resuspended in 0.25 M sucrose. 1 mM EDTA, 1 mM tris/HCl pH 7.3. Oxygen uptake was measured polarographically with a Clark electrode. Respiratory rates, respiratory control ratios (RCR) and ADP/O ratios were calculated according to Chance and Williams (10).

The rate of phosphorylation was measured as follows: mitochondria (0.1 mg of protein) were incubated in 1 ml of medium containing 250 mM sucrose, 20 mM Tris, 8 mM $MgCl_2$ and 10 mM H_3PO_4, pH 7.3. The medium was supplemented with glutamate (10 mM) plus 1 mM malate or succinate (10 mM) as substrates. The reaction was initiated by the addition of 120 nmol ADP. The generation and extrusion from the mitochondria of ATP were recorded as production of NADPH by adding to the medium 50 mM glucose, 4 units hexokinase, 80 nM NADP, and 0.35 units glucose-6-phosphate dehydrogenase. Generation of NADPH was followed at 340 nm in a Pye unicam spectrophotometer, the absorbance change being continuously recorded. The reaction was linear for at least five minutes. Adenine nucleotides present in freshly prepared mitochondria were determined in a perchloric acid extract as described in (1).

The activity of glycerophosphate oxidase was assayed polarographically in the whole homogenate (11). ATPase activity was determined at 30°C in a reaction system consisting of 200 mM sucrose 1 mM EDTA, 10 mM ATP, 50 mM tris/HCl, pH 7.4 and about 2 mg of mitochondrial protein, in a final volume of 1.5 ml. In some experiments 2 mM $MgCl_2$, 100 μM DNP or 100 μM DNP plus 20 μg oligomycin were added. The reaction was initiated by ATP and terminated by the addition of trichloroacetic acid (final concentration 5%). After removing precipitated protein, the release of ionorganic phosphate (Pi) was determined in aliquots by the method of Sumner (12).

The activity of adenine nucleotide translocase was measured by determining the translocation of ^{14}C-ADP into the mitochondria according to the method described by Lerner (13) as modified by Gordon (14). Mitochondrial swelling was assayed essentially as described by Cederbaum et al (15). Statistical significance between comparable groups was determined by the Student's t test.

3. RESULTS AND DISCUSSION

The oxidation of site I substrates (glutamate, malate) by liver mitochondria isolated from animals treated with glucose or ethanol and saline or adenosine was studied. The administration of adenosine or ethanol increased the rate of oxygen uptake in state 3 (Table I). Administration of both agents (ethanol + adenosine) produced and additive response (Table I). No significant changes in state 4 rates were observed and therefore the RCR was higher in mitochondria isolated from animals treated with adenosine or ethanol as compared to the control group (glucose + saline). The actions of adenosine and ethanol on the RCR were additive. Uncoupled respiration was also slightly increased by the administration of ethanol or adenosine (Table I) and the ADP/O was not modified by any treatment (Table I).

When succinate was used as substrate no clear cut effect of ethanol or adenosine was observed in any of the respiratory parameters measured (Table I). The oxidation of alpha-glycerophosphate by isolated mitochondria was unaffected by the administration of ethanol, slightly reduced by adenosine and significantly diminished (approx 35%) by treating the rat with both ethanol and adenosine (Table I). Consistent with these findings are the activities of hepatic alpha-glycerophosphate oxidase which were as follows: glucose + saline group 10.82 $\pm$ 1.39; glucose + adenosine group 7.56 $\pm$ 0.85; ethanol + saline 8.34 $\pm$ 1.11 and

ethanol + adenosine 6.98 ± 0.33 natoms O_2 min^{-1} mg $protein^{-1}$ (the results are the mean ± SEM of 3 determinations in each case).

The effects of adenosine and ethanol on the rate of phosphorylation by isolated mitochondria were also studied and the results are presented in Table II.

The rate of phosphorylation using glutamate plus malate as substrates was stimulated in mitochondria isolated from animals treated with either ethanol or adenosine, and the actions of ethanol and adenosine were additive, (Table II) reflecting the effects observed previously (Table I). Unexpectedly, ethanol treatment increased the rate of phosphorylation with succinate as substrate. Adenosine treatment had very little effect on either the basal rate of phosphorylation or the action of ethanol when succinate was used as substrate. (Table II).

It has been proposed that the translocation of adenine nucleotides is rate limiting for oxidative phosphorylation (16) and that the activity of the translocator plays a role in the coordination of the redox states of cytosol and mitochondria (17). However, the importance of this translocator has been questioned (18,19). Under our conditions no significant modifications in the activity of the enzyme were observed (Table III). An increase in the adenine nucleotide pool in the mitochondria has been claimed as responsible for the effects of hormones on mitochondrial function (20,22). Under our conditions, no significant change in the amount of adenine nucleotides was found after any of the treatments (data not shown). Modifications in the activity of the mitochondrial ATPase have also been observed after hormonal stimulation (20,22). The activity of the enzyme was assayed under basal and stimulated conditions. No effect of any of the treatments was observed (Table III).

Alterations in the permeability of rate limiting factors could play a role in the effects of the nucleoside on oxidative phosphorylation. However, neither the activity of the adenine nucleotide translocator (permeability to ADP) nor the ATPase (protons) were significantly affected. In an effort to detect major changes in the permeability of the inner mitochondrial membrane, the ability of mitochondria to swell in the presence of amonium salts was assayed. No significant changes were observed in response to any of the treatments. The uptake of oxygen by sonicated mitochondria and submitochondrial particles, in which no permeability barrier exists, was studied. The oxidation of NADH was stimulated in preparations from adenosine-treated animals (Table IV) and no effect on the oxidation of succinate was observed (Table V), in agreement with the results obtained with intact mitochondria (Tables I and II). The data rule out the participation of mitochondrial matrix factors or metabolite transport in this action of the nucleoside and suggest that a change in the mitochondrial inner membrane is produced.

The administration of adenosine to fasted rats results in a change in the hepatic mitochondrial function, persistent even after the isolation procedure. The effect seems to be localized in the respiratory chain and is much more easily observable with site I substrates. The physiological relevance of this findings remains to established. However, it seems to be linked to the following actions of the nucleoside: a) the increase in the energy parameters (1,6,7), b) the increase in the mitochondrial NAD/NADH ratio (8) and c) the increase in ethanol oxidation and amelioration of the ethanol-induced fatty liver (1,2).

We previously suggested that the transfer of reducing power from cytosol

to mitochondria and its oxidation in the mitochondria may be a significant part of the mechanism by which adenosine increases ethanol oxidation and ameliorates the cytoplasmic redox state due to ethanol oxidation (1,2). The present data lend support to that suggestion. In addition, it suggests that the transfer of reducing power is not through the α-glycerophosphate shuttle since the activity of glycerophosphate oxidase is decreased.

Ethanol administration significantly stimulated in the oxidation of some substrates and the rate of phosphorylation. It has been previously observed that addition in vitro of ethanol at low concentrations can enhance some mitochondrial functions such as Ca^{2+} uptake and state 3 oxygen uptake, probably as the result of an increase in membrane fluidity (23). This action may be related to the effects of ethanol administration observed in the present studies.

Recently it has been reported that the action of some hormones, such as glucagon and epinephrine (alpha-adrenergic action), involves stimulation of some mitochondrial functions (20,22). The present results suggest that adenosine, shares this property.

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Table I. EFFECT OF ETHANOL OR GLUCOSE AND ADENOSINE OR SALINE ON OXYGEN UPTAKE BY HEPATIC MITOCHONDRIA

Oxygen uptake was measured polarographically in the absence (state 4) or presence (state 3) of 0.5 mM ADP. Medium composition as follows: 250 mM Sucrose 20 mM Tris, 8mM $MgCl_2$ and 10 mM H_3PO_4 pH 7.3. Substrates: 10 mM in the case of glutamate or succinate, 1 mM malate. DNP was used at the final concentration of 100 μM. Experiments were performed with 2 mg of mitochondrial protein in a final volume of 3 ml. RCR, respiratory control ratio; P/O = ADP/O ratio. Results except for RCR and P/O are expressed as natoms O/min^{-1} $protein^{-1}$ and are the mean ± the standard error of four animals for glycerophosphate and 8 animals for succinate and glutamate-malate.

Respiratory Parameter	Treatment: Glucose + Saline	Glucose + Adenosine	Ethanol + Saline	Ethanol + Adenosine
Glutamate-Malate				
State 4	10.08 ± 0.66	9.62 ± 0.96	10.63 ± 0.96	9.87 ± 0.94
State 3	55.69 ± 2.83	64.52 ± 2.41[a]	65.83 ± 3.07[a]	73.36 ± 4.07[b]
DNP	67.15 ± 3.51	74.98 ± 4.38	81.47 ± 4.55	79.16 ± 4.49
RCR	5.52	6.71	6.19	7.43
P/O	2.70 ± 0.10	2.93 ± 0.10	2.75 ± 0.06	2.85 ± 0.09
Succinate				
State 4	18.34 ± 0.94	18.35 ± 1.16	20.69 ± 1.84	19.33 ± 1.69
State 3	81.61 ± 4.56	98.08 ± 6.40	87.39 ± 5.18	93.63 ± 5.96
DNP	106.48 ± 6.86	109.22 ± 7.50	104.99 ± 5.20	104.93 ± 5.95
RCR	4.45	5.34	4.22	4.84
P/O	1.73 ± 0.05	1.98 ± 0.06	1.87 ± 0.05	1.94 ± 0.05
Glycerophosphate				
State 4	20.08 ± 2.02	16.54 ± 0.84	18.76 ± 3.62	16.99 ± 2.66
State 3	25.74 ± 1.59	20.75 ± 2.19	22.85 ± 5.48	16.86 ± 1.55[a]
RCR	1.28	1.25	1.22	0.99

[a] p 0.05 compared to the glucose + saline group

[b] p 0.005 compared to the glucose + saline group

Table III. EFFECT OF ETHANOL OR GLUCOSE AND ADENOSINE OR SALINE ON THE ACTIVITY OF ADENOSINE TRIPHOSPHATASE AND ADENINE NUCLEOTIDE TRANSLOCASE.

DNP dinitrophenol. The results are expressed as the mean ± S.E.M. with the number of determinations in parenthesis.

Enzyme activity	Treatment			
	Glucose + saline	Glucose + adenosine	Ethanol + saline	Ethanol + adenosine
ATPase nmol-Pi/min^{-1} mg prot.$^{-1}$				
Basal	10.69 ± 0.69 (4)	12.55 ± 3.02 (4)	11.53 ± 1.58 (4)	11.17 ± 1.52 (4)
Mg^{++}	22.97 ± 2.83 (4)	24.25 ± 4.67 (4)	30.01 ± 3.51 (4)	26.40 ± 4.11 (4)
DNP	219.09 ± 23.55 (4)	233.23 ± 22.98 (4)	241.04 ± 29.52 (4)	228.62 ± 28.53 (4)
DNP + Oligomycin	9.67 ± 5.07 (3)	13.23 ± 5.35 (3)	16.88 ± 1.64 (3)	14.13 ± 3.14 (3)
Adenine Nucleotide Translocase cpm/mg protein	631.18 ± 66.84 (5)	972.28 ± 143.05 (6)	701.47 ± 89.62 (6)	873.01 ± 118.45 (6)

Table IV. EFFECT OF ETHANOL OR GLUCOSE AND ADENOSINE OR SALINE ON OXYGEN UPTAKE BY MITOCHONDRIAL PARTICLES.

The results are expressed as natoms of oxygen min^{-1} mg $protein^{-1}$ and represent the mean ± S.E.M. of at least six determinations. Medium composition: 225 mM sucrose, 20 mM Tris-HCl pH 7.3, supplemented with either 1 mM NADH or 5 mM succinate.

Preparation	Treatment			
	Glucose + saline	Glucose + adenosine	Ethanol + saline	Ethanol + adenosine
Sonicated Mitochondria				
NADH	82.09 ± 8.29	110.50 ± 6.43[a]	100.77 ± 11.24	114.53 ± 10.07[b]
Succinate	72.61 ± 7.05	87.66 ± 9.37	74.28 ± 8.20	77.17 ± 6.51
Submitochondrial particles				
NADH	189.31 ± 17.79	274.52 ± 14.93[c]	220.15 ± 9.64	278.24 ± 35.39[b]
Succinate	144.17 ± 16.65	142.17 ± 13.83	136.61 ± 13.18	137.24 ± 6.85

[a] $p < 0.025$ compared to the glucose + saline group
[b] $p < 0.05$ compared to the glucose + saline group
[c] $p < 0.005$ compared to the glucose + saline group