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ALGUNOS CAMBIOS BIOQUIMICOS EN EL MUSCULO CARDIACO ISQUEMICO Y SU RELACION CON LA ACTIVIDAD NICOTINAMIDA ADENINA DINUCLEOTIDO GLICOHIDROLASA

por

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Esta tesis es un resumen de las siguientes publicaciones referidas en el texto:

1. Calva, E., A. Mújica, R. Núñez, K. Aoki, A. Bisteni and D. Sodi-Pallares: Mitochondrial biochemical changes and glucose-KCl-insulin solution in cardiac infarct. Am. J. Physiol. 211:71-76, 1966.

2. Calva, E., A. Trillo, R. Núñez, K. Aoki y D. Ariza: Relaciones entre los cambios bioquímicos y ultraestructurales en mitocondrias del corazón con infarto experimental. Arch. Inst. Cardiol. Méx. 39:696-712, 1969.

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INTRODUCCION -

El conocimiento y la caracterización cinética de la enzima NAD glicohidrolasa del músculo cardiaco tuvo como antecedente, en nuestro laboratorio, el estudio del infarto agudo experimental del miocardio.

En el corazón humano se presentan tres formas clínicas de cardiopatía coronaria: la cardiopatía arterioesclerótica, la angina de pecho y el infarto del miocardio. Las tres son consecuencia de una misma causa aparente: la disminución del flujo sanguíneo por el estrechamiento de las arterias coronarias debido a ateroesclerosis. La forma más importante es el infarto del miocardio porque de esta enfermedad muere el mayor porcentaje de enfermos con cardiopatía coronaria y constituye la causa de muerte más frecuente en los países industrializados, ocupando el - cáncer el segundo lugar. Además esta enfermedad está afectando con más frecuencia cada vez jóvenes, particularmente del sexo masculino.

En el infarto del miocardio, conocido también como necrosis isquémica, el daño cardiaco es ocasionado casi siempre por la oclusión completa de una de las arterias coronarias, en sitios previamente estenosados por lesiones ateroescleróticas.

El estudio de los cambios estructurales, funcionales y bioquímicos que ocurren en los órganos afectados por la enfermedad, es importante para entender cómo se establece y evoluciona el daño patológico y consecuentemente cuáles son los datos más va-

liosos para el diagnóstico y pronóstico y cuáles deben ser las - medidas preventivas y terapéuticas más razonables. Los estudios bioquímicos que hemos hecho en el tejido cardiaco del perro con necrosis isquémica, provocada por la ligadura de la rama descendente anteior de la coronaria izquierda, nos han permitido establecer algunas correlaciones entre los trazos electrocardiográficos, las alteraciones citológicas y algunos cambios bioquímicos y sobre todo, nos han revelado la participación de la enzima NAD glicohidrolasa cardiaca en uno de los cambios más aparentes que ocurren en la fase aguda de esta isquemia: la disminución del contenido de los dinucleótidos de nicotinamida; la cual a su vez, puede ser la causa de la alteración en el funcionamiento de la cadena respiratoria y en los procesos de la fosforilación oxidativa.

METODOLOGIA -

Los trazos electrocardiográficos sirvieron de base para calificar el grado y la extensión del daño histológico. El registro se hizo a través de un electrodo explorador aplicado en diferentes puntos de la superficie epicárdica de la cara anterior del ventrículo izquierdo (tejido lesionado) y en la porción superior de la cara posterior del mismo ventrículo (tejido control). Los trazos fueron tomados 30 min después de ligar la rama descendente anterior de la arteria coronaria izquierda y 5 min - antes de extraer el corazón. Los signos electrocardiográficos que sirvieron para calificar cualitativamente el daño fueron -

fundamentalmente; el desnivel del segmento ST y la alteración del complejo QRS (1,2).

La fracción subcelular estudiada fue la mitocondrial que se aisló siguiendo el método de Hagihara modificado por nosotros, particularmente para obtener las mitocondrias de la porción dañada del corazón (1,2).

La extirpación del corazón, y consecuentemente la iniciación de la preparación de la fracción mitocondrial, se hizo a los 30 min, a las 4, 8 y 12 horas, respectivamente, después de la ligadura de la arteria coronaria (1,2).

La preparación mitocondrial estudió la capacidad de síntesis del ATP, incubándola con uno de los dos sustratos típicos: el D,L-β-hidroxibutirato o el succinato. Con los datos obtenidos se calculó la velocidad del consumo de oxígeno, la relación P/O y el índice del control respiratorio. Además se midió el contenido total de los dinucléotidos de nicotinamida y adenina de la fracción mitocondrial, antes y después de la determinación del consumo de oxígeno. También se determinó el contenido del ion K. Por otra parte, el estudio involucró la observación con el microscopio electrónico de las preparaciones del tejido cardiaco tanto de la zona control como de la afectada por la isquemia y de las preparaciones mitocondriales de ambas zonas (1,2).

Discusion –

En la fracción mitocondrial del tejido cardiado de la zona

control la velocidad de consumo de oxígeno fue de 26 a 52 nátomos por min por mg de proteína con el β-hidroxibutirato como sustrato y de 64 a 84 con el succinato (2). La relación P/O con el primer sustrato fue de 1.92 a 2.41 y con el segundo de 2.16 a 2.38, o sea, en ambos casos, entre 2.0 y 2.5. El contenido total de los dinucléotidos de nicotinamida y adenina en la fracción mitocondrial recién obtenida fue de 5.50 a 6.86 µmoles por gramo de proteína. Las preparaciones mitocondriales recobradas al final de las lecturas manométricas, presentaron una disminución de 10 a 15% del contenido de dinucleótidos con respecto a la concentración original, independientemente del sustrato con el que fueron incubadas (1,2).

En las mitocondrias del tejido cardiaco con necrosis, la velocidad del consumo de oxígeno bajó 25% a las 8 horas de la isquemia y casi fue cero a las 12 horas de esta, en las incubaciones hechas en presencia del β-hidroxibutirato y, cosa notable, se conservó igual a las preparadas del tejido control cuando el sustrato fue el succinato (2). La eficiencia de la fosforilación oxidativa, juzgada por la relación P/O, fue de 1.65 a los 30 min y bajó hasta 0 en las muestras de las 12 horas de necrosis, incubadas con β-hidroxibutirato. Lo mismo sucedió, aunque el descenso fue más tardío, cuando el estudio se hizo incubando las mitocondrias en presencia de succinato. La diferencia en la evolución del cambio en los valores P/O con uno y sustrato, parece indicar una mayor labilidad del Sitio I de la fosforilación oxidativa en la cadena respiratoria, con respecto al Sitio II. Las

preparaciones mitocondriales del tejido isquémico mostraron una baja progresiva del contenido total de los dinucléotidos de nicotinamida y adenina, baja que llegó a ser del 40% del valor inicial a las 4 horas y del 90% a las 12 horas. Además estas mitocondrias perdían un gran porcentaje de su contenido de nucleótidos durante la incubación en el respirómetro de Warburg, pérdida que era claramente mayor que la que ocurría en las mitocondrias del tejido control (1,2).

Las células cardiacas del tejido control mostraban abundantes gránulos de glucógeno en los espacios intermiofibrilares y sobre las miofibrillas, así como alrededor del núcleo; las miofibrillas estaban contraídas y no se apreciaba la banda I; las mitocondrias tenían variadas formas, eran del tamaño del sarcómero y ocupaban la mayor parte de los espacios interfibrilares y de los espacios paranucleares, así como el espacio subyacente a la membrana plasmática; las crestas de estos organelos eran paralelas entre sí y llenaban el espacio intramitocondrial, siendo escasa la matriz y moderadamente densa a los electrones. En las preparaciones de la fracción mitocondrial, las mitocondrias aisladas del tejido control, aparecían de corte circular todas ellas y con sus crestas muy compactas (2).

A los 30 min de la oclusión arterial el aspecto del tejido cardiaco a simple vista era casi normal, mientras que a las 12 horas el cambio era visible en todo el espesor de la pared cardíaca: la necrosis aparente era transmural más que subepicárdica,

como ocurría en las primeras horas. El porcentaje de mitocondrias dañadas que aparecían en las preparaciones de estos organelos, aumentaba en razón del tiempo de oclusión de la arteria (2).

En las preparaciones que se estudiaron del tejido infartado, las alteraciones estructurales no aparecían progresivamente a lo largo del tiempo, ya que podían verse grandes cambios en un corte casualmente seleccionado después de unos cuantos minutos de isquemia y pocas alteraciones en muestras de tejido con mayor tiempo de daño; en otras palabras, dependía mucho de la muestra tomada al azar para este análisis; por esto en este tipo de estudios deberá observarse al microscopio un número mucho mayor de cortes para valorar mejor los resultados. Los datos más frecuentemente observados fueron: el ensanchamiento notable de los espacios interfibrilares, la desaparición de los gránulos de glucógeno, el desgarramiento transversal y longitudinal de las miofibrillas, el ensanchamiento de los espacios intramitocondriales y la ruptura de las crestas, llegando a verse el espacio intramitocondrial vacio o bien ocupado en parte por unas estructuras muy típicas formadas por restos de crestas agrupadas en haces relativamente muy densos a los electrones.

El contenido del total de los dinucleótidos de nicotinamida y adenina de la pared del ventrículo izquierdo normal del perro fue 1.007 ± .106 (promedio y error estándar) µmoles por gramo de tejido húmedo, valor que incluye .676 ± .050 de NAD, .276 ± .036

de $NADH_2$ .043 ± .014 de NADP y .032 ± .005 de $NADPH_2$. Estas cifras se obtuvieron en muestras de corazones perfundidos por las arterias coronarias con nicotinamida 0.5 M en solución de Ringer, inmediatamente después de ser extirpados (3).

En el tejido control de los corazones con infarto experimental, los valores del total y de cada una de las cuatro formas de estos dinucleótidos fueron similares a los del corazón normal del perro.

El contenido de nucleótidos de nicotinamída en la pared anterior y de la posterior, respectivamente, del ventrículo izquierdo, a los 30 min de la oclusión, fueron semejantes entre sí e iguales a los obtenidos en los corazones normales. A partir de la segunda hora de la ligadura se observó una disminución progresiva del contenido de estos nucleótidos y a las cuatro horas la pérdida del NAD y del NADP, tanto en sus formas oxidadas como reducidas, era de un 50%. A las 48 horas disminuyó 90% con relación a los valores de la zona control (3).

Se consideraron varias posibilidades para explicar la disminución de estas coenzimas en el tejido con infarto: la salida de las coenzimas al torrente circulatorio, su degradación *in situ* por alguna actividad enzimática del tejido o bien una deficiencia en su síntesis (3).

Para estudiar la primera posibilidad, fue necesario determinar los valores del NAD oxidado y del NAD reducido en la sangre arterial general y en la sangre venosa del seno coronario, donde

se supuso afluirían directamente los dinucleótidos del tejido dañado durante el infarto; no encontró diferencia en los niveles de ambas sangres al tiempo cero. La concentración de NAD fue 35 ± 6 µmoles de NAD por kg de sangre arterial con un hematocrito de 45%. Durante el infarto del miocardio el contenido de nucléotidos de nicotinamida permaneció igual en la sangre del seno coronario y en la sangre arterial y con valores semejantes a los iniciales. Sin embargo, no se puede descartar la posibilidad de que pudieran haber salido al torrente circulatorio en forma lenta y en pequeñas cantidades no detectables por el método utilizado y que ésto, aunado a la presencia de una NADasa en la sangre del perro, semejante a la descrita en otras especies, pudo haber impedido la observación de un aumento en el contenido de NAD en la sangre del seno coronario.

Al estudiar la segunda posibilidad, se demostró la presencia de una actividad enzimática que degrada al NAD, cuando se provoca la desorganización intracelular al fragmentar el tejido cardiaco normal con el homogeneizador. Por otra parte, los datos parecen indicar que la anoxia por sí sola no es el factor responsable de provocar la degradación de las coenzimas, ya que en fragmentos de tejido cardiaco normal incubados por 45 ó 60 min a 37°C, en los que existe anoxia tisular no disminuyó el contenido de NAD como sucedió durante la homogeneización.

En el tejido cardiaco del ventrículo izquierdo con dos horas de isquemia, encontramos que no sólo el contenido de NAD fue menor que en el tejido normal, sino que hubo un aumento equivalente de

nicotinamida. Todavía más cuando, los fragmentos de este tejido infartado se incubaron en las mismas condiciones que los del tejido normal, el contenido, ya de por sí bajo, de NAD, disminuyó más y la nicotinamida aumentó en cantidad equivalente.
Es posible que la desintegración intracelular *in vivo*, al desorganizar los compartimientos respectivos de la enzima y del NAD, propicie la acción de la enzima sobre el dinucleótido (4).

En la suspensión homogeneizada de corazón normal, la velocidad de degradación del NAD agregado a esta suspensión, fue semejante a la velocidad de degradación del NAD contenido en la propia preparación; la cual sugiere que la mayor parte del NAD intracelular se halla en forma libre, puesto que el NAD unido a proteínas es hidrolizado más lentamente por la enzima (4). Además, la cantidad degradada de NAD, fue equivalente a la cantidad formada de nicotinamida; dado que la relación del NAD desaparecido, medido con la deshidrogenasa láctica, y la nicotinamida formada, medida colorimétricamente, fue de 1.

La actividad enzimática que cataliza la degradación del NAD fue inhibida totalmente por la nicotinamida, cuando este inhibidor se agregó a la mezcla de incubación en la proporción de 4.5 mmoles por gramo de tejido.

En el tejido cardiaco normal la actividad NADasa, expresada como micromoles de NAD desaparecido en 15 minutos por gramo de tejido, osciló entre 7.3 y 13.4 y en tejido cardiaco isquémico los valores fueron semejantes (4).

El pH óptimo de esta actividad enzimática en preparaciones

homogeneizada de tejido cardiaco normal de perro, se encontró en una zona entre 6.2 y 7.6 cuando el sustrato fue NAD. Cuando se usó como sustrato NAD, NADP o NMN, siempre se liberó una cantidad de nicotinamida equivalente al nucleótido degradado, lo que permitió considerar a esta actividad enzimática como una NAD - glicohidrolasa específica, que actúa sobre el enlace N-β-glicosídico que une la nicotinamida a la ribosa, tanto en los dinucleótidos como en el mononucléotido.

Los valores de la K_m para el NADP y el NAD fueron 46 a 56 y 67 a 71 μM, respectivamente, en cambio para el NMN la K_m fue de 120 a 135 μM (5).

La nicotinamida y la hidrazida del ácido nicotínico actuaron como inhibidores no competitivos y los valores de la K_I fueron de 1.2 a 1.6 mM y de 0.24 mM a 0.25 mM, respectivamente, con NAD como sustrato. Cuando se incubaron las preparaciones en presencia de EDTA o bien de AMP, inhibidores característicos de la pirofosfatasa, no hubo inhibición y por el contrario, en el caso del AMP, se activó la degradación del NAD (5).

La actividad NAD glicohidrolasa de la suspensión homogeneizada del corazón del perro calentada primero a 50°C durante 15 min y medida después a 37°C, fue semejante a la de otra muestra de la misma preparación mantenida a 4°C y medida después a 37°C lo que demostró que el calentamiento modifica reversiblemente a la enzima.

La energía de activación calculada de una gráfica de Arrhenius, entre 10 y 50°C fue de 10 kcal (42kJ) por mol. La gráfica no -

presentó inflexiones que pudieran significar la presencia de varias enzimas o importantes cambios conformacionales de la enzima o del medio en que está anclada (5).

El fraccionamiento subcelular de la suspensión homogeneizada del corazón de perro, privada de la fracción nuclear, nos permitió demostrar los mayores porcentajes de la actividad en dos fracciones: la mitocondrial (17.3%) y la microsomal sedimentada a 17000 xg (44%). La fracción mitocondrial tuvo una actividad específica de 38 y la fracción de 17000 xg de 515 - nmoles de NAD degradado por 15 min por mg de proteína (6).

Los estudios de microscopía electrónica de la fracción - 17000 xg mostraron un material vesicular homogéneo sin contaminación aparente con restos mitocondriales o con mitocondrias - enteras.

En los estudios de las enzimas marcadoras se encontró que las actividades específicas de la ATPasa dependiente de Na^+ y $-K^+$, de la ATPasa Na^+ $-K^+$ $-Mg^{++}$ y de la fosfatasa ácida, respectivamente, eran mayores en la fracción de 17000 xg que en el citosol, en la fracción mitocondrial o en la microsomal de 105000 xg; lo que permitió considerar que la fracción de 17000 xg tenía un alto contenido de membranas plasmáticas, ya que la ATPasa - dependiente de Na^+ y K^+ se considera peculiar de esta estructura celular (6).

Para dilucidar si la actividad de NADasa presente en la - fracción mitocondrial era propia de las mitocondrias o se trataba

de una contaminación, se analizó la preparación a través de un gradiente discontinuo de sacarosa y se obtuvieron cuatro bandas, siendo las más puras la 1 y la 3. La microscopía electrónica mostró que la banda 1 estaba constituida por vesículas semejantes a las obtenidas en la fracción microsomal de 17000 xg y en ella se encontró la mayor actividad específica de NADasa (Fig. 1). La banda 3 contenía únicamente mitocondrias en buen estado y tuvo la menor actividad específica de NADasa y la mayor de citocromo oxidasa (Fig. 2). Estos hallazgos nos permitieron concluir que en la suspensión homogeneizada del tejido cardiaco del perro liberada de la fracción nuclear, la actividad NADasa se localiza en una fracción membranosa microsomal; sin embargo, no podemos excluir que esta fracción microsomal, constituida en su mayor parte por membrana plasmática, no esté contaminada con retículo sarcoplasmático.

Recientemente hemos iniciado el estudio de la actividad NADasa en la sangre humana (8), para poder valorar la participación que pudiera tener esta actividad en la degradación del NAD del tejido cardiaco isquémico, pues es sabido que durante la necrosis ocurre extravasación de algunos componentes sanguíneos al miocardio dañado.

CONCLUSION -

La NADasa del tejido cardíaco normal y del isquémico tienen la misma actividad específica. La ruptura de la integridad celular por medios mecánicos provoca la degradación total del NAD

celular por acción de la NADasa. En el tejido isquémico hay desorganización celular y disminuye la concentración de NAD, lo que probablemente afecta las reacciones en que participen las deshidrogenasas dependientes de NAD, como fue el caso de la ß-hidroxibutirato deshidrogenasa y consecuentemente, los procesos de fosforilación oxidativa concomitantes. Estos hechos ponen de manifiesto por primera vez la participación de la enzima NADasa en la patología bioquímica del infarto experimental del miocardio.

SUMMARY

Cardiac infarcts were produced in dogs by ligature of the anterior descending coronary artery and electrographic records were used to detect the ischemic area. Animals were sacrified 1/2,2,4,8,12 and 48 hours after arterial ligation. Mitochondria from the infarcted tissue showed a decrease in the P/O ratio and low oxygen consumtion with D,L-β-hydroxibutirate, whereas incubated with succinate the oxygen consumption occurred at normal rate but with a low P/O ratio.

The average level of adenine nicotinamide nucleotides in the damaged tissue decreased remarkably after the arterial occlusion. However, the concentration of NAD and $NADH_2$ before and after occlusion was the same in the coronary sinus blood. The ultrastructural damages observed in cardiac cell were dilation of intermyofibrillar spaces, disappearance of glycogen granules, rupture of myofibrils, mitochondrial swelling with disorganization and fragmentation of their cristae, lost of their granules and appearance of bundles of cristae.

NAD was rapidly hydrolyzed with the formation of stoichiometric amounts of nicotinamide when the normal cardiac tissue was mechanically disrupted. Nicotinamide completely inhibited NAD degradation.

The activation energy of NAD glycohydrolase of homogenized suspensions of the normal dog heart was 10 kcal mol^{-1} in the range 10 to 50°. The K_m values were 70 μM for NAD, 50 μM for

NADP and 130 μM for NMN.

The NADase appeared to be localized mainly in the membranous fractions. The 17000xg fraction showed the greatest specific activities of Na^+-K^+-ATPasa, acid phosphatase and K^+-activated phosphatase, i.e., it had a high proportion of plasmatic membranes. During the infarction process the structural disorganization determines the condition for a closer relation between the NADase which is localized in the membrane and the NAD distributed mainly in the cytosol.

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Mitochondrial biochemical changes and glucose-KCl-insulin solution in cardiac infarct[1]

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CALVA, EDMUNDO, ADELA MÚJICA, ROSARIO NUÑEZ, KAZUKO AOKI, ABDO BISTENI, AND DEMETRIO SODI-PALLARES. *Mitochondrial biochemical changes and glucose-KCl-insulin solution in cardiac infarct.* Am. J. Physiol. 211(1): 71–76. 1966.—Cardiac infarcts were produced in dogs by ligature of the anterior descending coronary artery and followed by electrocardiographic records. Animals were sacrificed ½, 4, 8, and 12 hr after the arterial occlusion. One group received glucose solution and another polarizing solution (glucose-KCl-insulin). Mitochondria prepared from normal and infarcted tissue were incubated with succinate or β-hydroxybutyrate. Oxygen consumption was measured manometrically, P/O ratio by phosphate uptake, nicotinamide adenine nucleotides fluorometrically, and potassium by flame photometry. In dogs receiving glucose solution a decrease in the P/O ratio appeared early followed by a loss of potassium and nicotinamide nucleotides; a low oxygen consumption with β-hydroxybutyrate was detected, whereas succinate was oxidized at a normal rate; mitochondria easily lost their nicotinamide nucleotides during incubation. Therefore, infarcted tissue mitochondria appear to have a defect in the phosphorylative reactions followed by a blockage of the respiratory chain. A protective action of the polarizing solution against all these changes was evident except for the nucleotide content, where its effect was minor.

mitochondria; sarcosomes; heart; myocardial infarction; oxygen consumption; manometric method; β-hydroxybutyrate; succinate; P/O ratio; nicotinamide adenine nucleotides; mitochondrial potassium; dogs; coronary artery occlusion; electrocardiogram

IN A PREVIOUS PAPER (2) we reported that mitochondria from cardiac tissue prepared 12 hr after occlusion of the anterior descending coronary artery showed no oxidative phosphorylation when the dogs had received glucose solution, whereas those isolated from dogs treated with "polarizing solution" maintained their values within normal range. Earlier, Sodi-Pallares et al. (8) had described a loss of intracellular potassium, and Govier (4) a breakdown of coenzyme I in infarcted myocardial tissue. In addition, Gamble (3) has suggested an interdependence between oxidative phosphorylation and potassium retention.

The present work was undertaken to extend our previous observations to mitochondria isolated at different times after the occlusion of the artery. Measurements of total nicotinamide adenine nucleotides and potassium in mitochondria are included to discuss their relation with oxidative phosphorylation reactions, in an attempt to understand the protective action of the polarizing solution.

MATERIALS AND METHODS

Animals

The handling of the dogs and the surgical technique were similar to those described previously, except that the intravenous solution was started at the time of the arterial occlusion and not 3 hr earlier (2). Twenty-two animals received 10% glucose solution (untreated infarcted dogs) and twenty-four more (treated infarcted dogs) received the polarizing solution (potassium chloride 40 mmoles and insulin 20 U/liter in 10% glucose solution). The animals in these series were sacrificed ½, 4, 8, and 12 hr after the total occlusion of the anterior descending coronary artery. Nine noninfarcted, sham-operated dogs were maintained for 3 hr and received either one of the two solutions. Finally, 14 dogs sacrificed by electric shock served as noninfarcted controls (some of the infarcted dogs were prepared by Dr. D. Ariza).

Electrocardiographic Tracings

Unipolar tracings were taken 30 min after the ligature of the artery and shortly before excising the heart from the animal (2). Records were obtained also from the sham-operated group at the begining and at the end of

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TABLE 1. *P/O ratios with β-hydroxybutyrate as a substrate*

Electrocuted	Noninfarcted Dogs		Infarcted Dogs*							
	Sham Operated		Untreated				Treated			
	Untreated	Treated	½	4	8	22	½	4	8	22
			Control myocardial tissues							
2.47 ±0.15 (14)	2.42 ±0.40 (4)	2.40 ±0.10 (5)	1.92 ±0.31 (4)	2.35 ±0.15 (6)	2.11 ±0.27 (3)	2.41 ±0.07 (8)	2.01 ±0.17 (4)	2.66 ±0.09 (8)	2.15 ±0.09 (5)	2.48 ±0.07 (7)
			Infarcted myocardial tissues							
			1.65 ±0.46 (5)	1.31 ±0.46 (6)	0.61 ±0.35 (4)	0 (4)	1.99 ±0.21 (4)	2.32 ±0.14 (6)	1.14 ±0.59 (3)	1.94 ±0.36 (3)

Values are means ± SE, with number of dogs in parentheses. * Time (hr) after ligation of the descending coronary artery.

the surgical handling (two dogs which showed no electrocardiographic evidence of infarct 30 min after the arterial occlusion were not included).

Mitochondrial Preparations

Heart mitochondria were isolated as described before (2) except that the extraction medium contained 2 mM adenosine triphosphate (ATP). With the addition of this nucleotide we obtained well-formed pellets in all our preparations even in those from untreated infarcted animals (2).

Oxidative Phosphorylation

Oxygen consumption was measured manometrically in 15-ml Warburg vessels with air as the gas phase. After 10 min at 30 C the reaction was initiated by tipping in the phosphate trap from the side arm. For inorganic phosphate determination (5) portions of 1.0 ml of the reaction mixture from two similarly prepared flasks were transferred to test tubes containing 0.32 ml 21.3% trichloroacetic acid: one just after the initial equilibration period, and one at the end of the 20-min manometric reading. The transfer and the mixing of the samples were accomplished in exactly 30 sec. Supernatants fluids were obtained after centrifugation at 5,000 *g* for 5 min at 0 C. ATP formation was assumed to be equal to the decrease in inorganic phosphate.

The phosphorylation medium was arranged as follows to have final molarities similar to those used (2) in the polarographic method: 284 mM D-mannitol, 95 mM sucrose, 25 mM tris buffer (tris(hydroxymethyl)amino methane) (Sigma Chemical Co. 7–9) and 12.6 mM KCl. For convenience, NaH_2PO_4 and $MgSO_4$ were changed to 17.3 and 2.6 mM, respectively, and the whole mixture adjusted to pH 7.2 at room temperature.

The main compartment of the flasks contained: 2.3 ml of the phosphorylation medium, 0.5 ml mitochondrial suspension with 4–12 mg protein, and 0.1 ml 0.2 M DL-β-hydroxybutyrate (Sigma Chemical Co.) or succinate (Eastman) at pH 7.0. In the side arm 0.2 ml of the phosphate trap was placed: 250 mM glucose, 7.5 mM adenosine diphosphate (ADP) and 150 units hexokinase (Sigma Chemical Co.) adjusted to pH 6.8 at room temperature.

Mitochondrial Total Nicotinamide Adenine Nucleotides[2]

Total nicotinamide adenine nucleotides (NAN), i.e., NAD, NADH, NADP, and NADPH were determined with an Aminco spectrophotofluorometer by the method of Lowry et al. (6). The extraction of the nucleotides from mitochondria was carried out by heating a 0.25-ml sample of the original mitochondrial preparation at 85 C for 5 min in 1.0 ml 50 mM tris, pH 8.2. The mixture was then quickly cooled and centrifuged at 8,000 *g* for 10 min at 0 C. Portions of 0.09 ml of the supernatant material were taken for analysis and the reduced nucleotide forms oxidized with hydrogen peroxide. Readings corrected with a suitable blank were referred to samples containing 1, 2, and 3 mμmoles NAD (Sigma Chemical Co.), respectively.

In some experiments the nucleotide content of the mitochondria was measured after the initial equilibration period and at the end of the 20-min manometric reading. The reaction mixture from the Warburg vessels was centrifuged at 8,000 *g* for 10 min at 0 C, and the mitochondrial pellet resuspended in 0.4 ml mannitol sucrose extraction medium.

Potassium in Mitochondria

Potassium was determined by flame photometry after digestion of 0.5 ml of the original mitochondrial preparation with 1.0 ml 1:10 HNO_3 and 0.2 ml 1:2 $HClO_4$. The pale yellow digest was quantitatively transferred to a volumetric tube containing 0.040 ml 5.000 g lithium/liter ($LiNO_3$, Mallinckrodt), and the volume was completed to 2 ml with glass double-distilled water. Blanks were prepared with extraction medium and the readings carried out in a Baird flame photometer by the internal standard method against 0.4 mM KCl (Mallinckrodt).

[2] Dr. William Cieplinski collaborated in these studies and presented part of them elsewhere (Tesis Profesional Univ. Nac. Aut. Méx. Fac. Med., 1965).

TABLE 2. *P/O ratios with succinate as a substrate*

Noninfarcted Dogs			Infarcted Dogs*							
Electrocuted	Sham operated		Untreated				Treated			
	Untreated	Treated	½	4	8	22	½	4	8	22
Control myocardial tissues										
2.33 ±0.06 (10)†	2.31 ±0.11 (4)	2.28 ±0.08 (5)	2.16 ±0.10 (5)	2.28 ±0.10 (6)	2.38 ±0.05 (3)	2.28 ±0.05 (8)	2.17 ±0.09 (4)	2.33 ±0.05 (7)	2.10 ±0.10 (5)	2.31 ±0.03 (7)
Infarcted myocardial tissues										
			1.87 ±0.18 (5)	1.38 ±0.25 (4)	1.55 ±0.21 (3)	0.26 ±0.19 (4)	2.13 ±0.05 (4)	2.19 ±0.12 (4)	1.61 ±0.28 (3)	1.89 ±0.28 (2)

Footnote same as Table 1.

Protein Determination

Protein was measured by the Folin-Ciocalteu method (7) using crystallized bovine plasma albumin (Armour) as a reference. A portion of 0.1 ml original mitochondrial preparation was solubilized with 0.1 ml 10% sodium deoxycholate and the volume completed to 3.0 ml with water.

RESULTS

Oxidative Phosphorylation

Control tissue. Mitochondrial preparations from nearby control areas of the left ventricular muscle of untreated dogs sacrificed at different times after arterial occlusion showed mean values for P/O ratios of each group to be from 1.92 to 2.41 with β-hydroxybutyrate (Table 1) and from 2.16 to 2.38 with succinate (Table 2) as substrate. These values did not differ significantly (Student-Fisher *t* test) from each other or from those obtained with dogs electrocuted or sham operated. Likewise, no difference was noted with the mitochondria from control areas of the group of treated infarcted dogs.

Infarcted tissue. A decrease in the P/O ratios of mitochondria from infarcted areas of untreated dogs with each one of the two substrates was observed as early as 30 min after the arterial occlusion. In the later samples the ratios continued to decrease progressively when they were estimated with β-hydroxybutyrate as a substrate (Table 1), whereas with succinate (Table 2) those preparations from the 4th and the 8th hr showed values similar to the ones observed at 30 min. Phosphorylation was not detected in the 12-hr sample when β-hydroxybutyrate was in the incubation mixture, whereas low values were obtained when succinate was the substrate added. Insofar as the results with the polarizing solution are concerned, P/O ratios with mitochondria from infarcted tissue of treated dogs were higher than those obtained with mitochondria from infarcted tissue of untreated dogs, irrespective of the substrate present in the incubation mixture; these differences were particularly remarkable at 12 hr (Tables 1 and 2).

Oxygen Consumption Rate

Control tissue. In all the experiments mitochondria consumed more oxygen (μatoms/min per mg protein) with succinate as an exogenous substrate than with β-hydroxybutyrate. Values for the control mitochondria of the various groups of dogs did not change significantly with either type of intravenous solution, with the surgical handling, or the method used to sacrifice the animals.

Infarcted tissue. Mitochondria of the infarcted area incubated with β-hydroxybutyrate, both from treated and untreated dogs, showed a similar low oxygen consumption rate at 4 and at 8 hr, but after 12 hr mitochondria from animals which received the polarizing solution remained as high as the 8-hr samples, whereas those from untreated dogs did not consume oxygen.

With succinate the mitochondria of the infarcted area did not show a fall in the oxygen consumption rate at any time regardless of the solution injected into the animal.

Content of NAN in Mitochondria and Their Loss During Incubation

Control tissue. Heart mitochondria freshly prepared from untreated dogs showed a NAN content from 5.50 to 6.86 μmoles/g protein. Similar values were determined in sarcosomes from either treated, sham-operated, or electrocuted dogs (Table 3). On the other hand, although this initial level decreased somewhat during the manometric measurements, the treatment of the animals with the polarizing solution seemed to protect the mitochondria against such losses (Table 4).

Infarcted tissue. A low content of NAN was observed following the 4th hr in mitochondria from infarcted untreated dogs, later becoming still lower. In contrast, the NAN content of mitochondria from infarcted treated dogs did not decrease as much, although a significant difference was observed with the control values at 8 and at 12 hr (Table 3). Mitochondria from infarcted tissue seemed to lose a greater percentage of the initial amount of NAN during incubation than those prepared

TABLE 3. *Nicotinamide adenine nucleotides content of mitochondria (μmoles/g protein)*

Electrocuted	Noninfarcted Dogs: Sham operated, Untreated	Noninfarcted Dogs: Sham operated, Treated	Infarcted Dogs*: Untreated, ½	Untreated, 4	Untreated, 8	Untreated, 12	Treated, ½	Treated, 4	Treated, 8	Treated, 12
Control myocardial tissues										
5.85 ±0.21 (13)	5.80 ±0.33 (4)	5.58 ±0.40 (5)	5.50 ±0.71 (5)	6.75 ±0.65 (6)	6.29 ±1.57 (2)	6.86 ±0.43 (8)	5.43 ±0.45 (4)	6.54 ±0.34 (8)	7.41 ±0.39 (5)	6.61 ±0.35 (7)
Infarcted myocardial tissues										
			6.03 ±0.92 (5)	4.76 ±0.61 (6)	0.96 ±0.73 (3)	0.71 ±0.22 (4)	6.47 ±0.25 (4)	6.55 ±0.41 (6)	2.69 ±0.50 (3)	3.32 ±0.66 (4)

Footnote same as Table 1.

TABLE 4. *Variations in the level of nicotinamide adenine nucleotides in mitochondria from control tissue*

	Untreated Dogs*: ½	Untreated Dogs*: 4	Untreated Dogs*: 8	Untreated Dogs*: 12	Treated Dogs*: ½	Treated Dogs*: 4	Treated Dogs*: 8	Treated Dogs*: 12
Initial content	6.30 (2)	6.79 (5)	5.86 (3)	7.51 (4)	5.34 (1)	6.54 (8)	7.96 (3)	6.99 (3)
With β-hydroxybutyrate†								
After equilibration	3.84 (2)	5.99 (4)	5.11 (3)	7.18 (3)	6.00 (1)	5.98 (6)	7.72 (3)	6.78 (3)
After incubation	2.96 (2)	5.23 (5)	4.83 (3)	6.16 (4)	4.95 (1)	5.96 (8)	7.27 (3)	6.25 (2)
With succinate†								
After equilibration	5.98 (1)	6.23 (5)	4.99 (3)	7.35 (2)	5.68 (1)	6.39 (5)	6.05 (1)	6.56 (3)
After incubation	6.39 (1)	5.42 (5)	4.94 (3)	5.93 (4)	5.32 (1)	6.22 (5)	7.82 (3)	7.00 (2)

Values (μmoles/g protein) are means with number of dogs in parentheses. * Time (hr) after ligation of the descending coronary artery. † Substrate in the incubation mixture for manometric measurements.

from control myocardial tissue. The losses were higher with β-hydroxybutyrate in the incubation mixture and increased in mitochondria from older infarcts. Mitochondria from dogs treated with the polarizing solution lost much less nucleotides than those from untreated animals (Table 5).

Potassium Content (Table 6)

Control tissue. The amount of potassium found in control mitochondria was from 117 to 155 μEq/g protein. There was no change either with nearby infarction or with the treatment.

Infarcted tissue. A loss of mitochondrial potassium after the occlusion of the artery was observed at 8 and at 12 hr. The treatment with polarizing solution maintained higher levels of mitochondrial potassium and this effect was more remarkable at 12 hr.

Electrocardiographic Records

Injury signs (elevation of RS-T) appeared in all the 30-min records from both groups of dogs, treated and untreated, included in this work. Nevertheless, ST-segment displacement and Q waves were less prominent in the electrocardiograms of dogs treated with the polarizing solution and taken 4, 8, and 12 hr after the arterial occlusion.

Low content of nicotinamide nucleotides and potassium in mitochondria and low P/O values always coincided with abnormal electrocardiographic signs. Of the three values, the lowering of the P/O ratios appeared to be more related to such electrocardiographic alterations. However, some dogs showed abnormalities in the electrocardiographic pattern, whereas the biochemical values were like those in the controls.

DISCUSSION

Mitochondria isolated from the anterior wall of the left ventricle after the ligature of the anterior descending coronary artery, and incubated either with β-hydroxybutyrate or succinate, showed an impaired phosphorylating efficiency soon after the occlusion of the vessel in dogs receiving only glucose. The P/O values measured with β-hydroxybutyrate as a substrate pro-

TABLE 5. *Variations in the level of nicotinamide adenine nucleotides in mitochondria from infarcted tissue.*

	Untreated Dogs*				Treated Dogs*			
	½	4	8	12	½	4	8	12
Initial content	7.54 (2)	4.67 (5)	0.96 (3)	0.49 (2)	6.32 (1)	6.38 (7)	4.38 (2)	2.80 (3)
With β-hydroxybutyrate†								
After equilibration	4.08 (2)	1.15 (3)	0.12 (3)	0.20 (1)	6.20 (1)	5.01 (5)		
After incubation	3.49 (2)	2.34 (5)	0.12 (3)	0 (2)	5.32 (1)	4.74 (7)	3.07 (3)	1.59 (2)
With succinate†								
After equilibration	5.96 (1)	4.90 (2)		0.25 (1)	6.30 (1)	5.77 (1)		
After incubation	6.38 (1)	4.75 (2)	0.92 (2)	0 (2)	5.00 (1)	5.50 (4)	3.65 (3)	

Footnote same as Table 4.

TABLE 6. *Potassium content of mitochondria (μEq/g protein)*

Noninfarcted Dogs			Infarcted Dogs*							
Electrocuted	Sham operated		Untreated				Treated			
	Untreated	Treated	½	4	8	12	½	4	8	12
Control myocardial tissues										
135 ±4 (10)	135 ±5 (4)	134 ±2 (4)	151 ±6 (5)	140 ±8 (6)	117 (1)	138 ±4 (8)	138 ±6 (4)	152 ±4 (8)	130 ±13 (5)	155 ±7 (7)
Infarcted myocardial tissues										
			161 ±4 (5)	123 ±16 (6)	92 ±2 (3)	19 ±5 (4)	152 ±5 (4)	148 ±9 (6)	97 ±5 (3)	133 ±2 (4)

Footnote same as Table 1.

gressively decreased and reached zero at 12 hr, whereas with succinate they were slightly lower than the control and remained roughly the same along the first 8 hr, then approached zero around the 12th hr. This difference could be due partly to the fact that the function of the respiratory chain linked to the oxidation of succinate was not affected during the 12 hr of the experiment, since oxygen consumption values similar to those observed with mitochondria from control tissue were maintained. On the other hand, the blockage of the respiratory chain which appeared when β-hydroxybutyrate was the substrate may have taken place at the coenzyme level, being the result of the loss of the nicotinamide nucleotides. In this respect, the extent of the NAN loss occurring during the manometric measurement seems to be more important than the level detected at the beginning. Therefore, the low P/O values observed in mitochondria from infarcted cardiac tissue seem to reflect an early defect in the phosphorylative reactions followed by a blockage of the respiratory chain at the step of the nicotinamide nucleotides.

According to Gamble's view (3) that there is an interdependence between energy-coupled reactions and mitochondrial potassium retention, it is probable that the loss of this cation was also due to impairment of the phosphorylative reactions.

In full agreement with our previous results (2), the dogs treated with the glucose-KCl-insulin solution showed electrocardiographic recovery signs in the cardiac infarcted area and the P/O values measured in mitochondria prepared from such tissue were like those from normal heart tissue. In addition, we now find that the mitochondria of this group of animals maintained a normal potassium content and initial levels of nicotinamide nucleotides intermediate between those found in the control group and the untreated infarcted group. These data show the protective action of the polarizing solution. Moreover, the mitochondria held their nucleotides better during the manometric measurements. In consequence, if the ligature of the artery leads to uncoupling of the phosphorylation reactions and a loss of potassium and nicotinamide nucleotides, the polarizing solution protects the phosphorylative system and through this the content of potassium and, less efficiently, that of total NAN.

It appears that the level of nicotinamide nucleotides

does not depend on the energy-linked functions exclusively, since oxidative phosphorylation could be carried out even with low levels of total NAN (see data for succinate, 8-hr untreated dogs and both substrates, 12-hr treated dogs).

P/O ratios measured with β-hydroxybutyrate or succinate in the reaction medium were similar and a little higher than those reported with the polarographic method (2). Such findings, which otherwise do not modify the general results, could be due to the modifications of the reaction mixture and to the conditions involved in each method (1). Finally, we found it was not necessary to inject glucose or polarizing solution 3 hr before the total occlusion of the artery as was done before (2).

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INVESTIGACION EXPERIMENTAL

RELACIONES ENTRE LOS CAMBIOS BIOQUIMICOS Y ULTRAESTRUCTURALES EN MITOCONDRIAS DEL CORAZON CON INFARTO EXPERIMENTAL

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LOS HALLAZGOS de Sodi-Pallares y su grupo[17] relativos a la evolución del infarto experimental del miocardio, en particular la recuperación de la amplitud de la contracción muscular en el área con infarto,[18] nos impulsaron a estudiar en el perro los cambios bioquímicos que ocurrieran en el tejido cardíaco después de ligar la rama descendente de la arteria coronaria izquierda. Nuestro interés inicial fue el de valorar la eficiencia de las reacciones de fosforilación oxidativa en mitocondrias preparadas del miocardio dañado;[1] sin embargo, como también se había demostrado que era menor el contenido de potasio intracelular[17] y el de coenzima I[5] y que había una interdependencia entre la fosforilación oxidativa y la retensión de potasio,[4] creímos que sería interesante medir el contenido de este catión y el total de nucleótidos de nicotinamida adenina en nuestras preparaciones mitocondriales, para poder discutir dichos hallazgos en función de otras alteraciones bioquímicas.[2] A partir de los primeros datos comprendimos la necesidad de estudiar los cambios no sólo a un tiempo fijo[1] sino a diferentes horas después de la ligadura de la rama arterial[2] y recientemente hemos obtenido, además, las imágenes al microscopio electrónico de las preparaciones mitocondriales, en un intento de establecer relaciones entre las alteraciones morfológicas y los cambios bioquímicos.

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METODO EXPERIMENTAL

Se utilizaron perros callejeros aparentemente normales de 14 a 18 kg de peso y los cuales fueron anestesiados con nembutal. Los animales recibieron continuamente una solución de glucosa al 10% a través de la vena femoral, a una velocidad de 3 ml/min y su respiración se mantuvo con una bomba Palmer unida a la cánula endotraqueal. A través de una incisión lateral izquierda del tórax se expuso el corazón ampliamente y luego de abrir el pericardio se disecó la arteria coronaria descendente izquierda cerca de su origen y se fue ocluyendo gradualmente hasta cerrarla por completo a los 30 minutos. Los trazos electrocardiográficos unipolares se tomaron a través de un electrodo indiferente conectado a la terminal central y un electrodo de exploración colocado sobre distintos puntos del epicardio de la pared anterior del ventrículo izquierdo y se registraron tanto a los 30 minutos después de la ligadura total de la arteria como a los 5 minutos antes de extraer el corazón del animal. Los perros fue-

ron sacrificados a las 12 horas en una primera serie de experimentos y 30 minutos, 4, 8 y 12 horas después en las últimas series, siempre y cuando los trazos electrocardiográficos de los primeros minutos hubieran mostrado signos de infarto. Se usaron dos porciones del ventrículo izquierdo, una fue la mayor parte de la pared anterior (tejido con infarto) y la otra la porción superior de la pared posterior, cuyo trazo electrocardiográfico fue normal en todos los casos (tejido de control). Las mitocondrias se aislaron de acuerdo con el método de Hagihara modificado[1] y la pastilla mitocondrial se obtuvo al centrifugar el sobrenadante, libre de restos celulares y de núcleos, durante 7 minutos a 7,000 x g. Al finalizar, las mitocondrias fueron resuspendidas en una solución de manitol, sacarosa, tris-(hidroximetil)aminometano y etilenodiaminotetraacetato, a pH 7.4.

En la primera serie de experimentos los cambios en la concentración de oxígeno se midieron polarográficamente con el electrodo de Clark. El índice de control respiratorio se calculó como la relación entre la velocidad del consumo de oxígeno después y antes, respectivamente, de la adición de adenosina difosfato (ADP) al medio de incubación de las mitocondrias y para establecer la relación entre la adenosina trifosfato (ATP) sintetizada y el oxígeno consumido, se determinó el ATP como glucosa-6-fosfato.[1]

En las series posteriores la cifra del consumo de oxígeno se obtuvo manométricamente. La relación P/O fue establecida de los valores del fosfato inorgánico y del oxígeno consumidos.[2] El contenido de nucleótidos totales de nicotinamida adenina se determinó con el espectrofluorómetro Aminco, por el método de Lowry y colaboradores[10] y las lecturas corregidas se refirieron a muestras que contenían 1, 2 y 3 milimicromoles de nicotinamida adenina dinucleótido[2] (NAD). El potasio mitocondrial se valoró por fotometría de flama después de digerir 0.5 ml. de la preparación mitocondrial con HNO_3 y $HClO_4$.[2] Las proteínas se determinaron por el método de Folin-Ciocalteu[13] en preparaciones solubilizadas con la adición de desoxicolato de sodio.

Para el estudio ultraestructural del músculo cardíaco se tomó un cilindro de tejido del área con infarto y de la de control, respectivamente, con un sacabocado número 3/16, tan pronto como el corazón se había extraído del perro. Cada cilindro se colocó inmediatamente en una solución al 6.25% de glutaraldehído en amortiguador de fosfatos[16] y se cortó en fragmentos pequeños de aproximadamente 1 mm^3. La fijación se prolongó por 90 minutos en el glutaraldehído a 4° y luego que las muestras se lavaron con dos cambios de amortiguador de fosfatos se postfijaron[12] durante una hora en tetróxido de osmio al 1%. Posteriormente los tejidos se deshidrataron en soluciones de alcohol etílico de concentración progresivamente creciente, se incluyeron[11] en Epon 812 y se cortaron en el ultramicrotomo Porter-Blum MT1. Finalmente, los cortes se montaron en rejillas de cobre, se tiñeron con solución de citrato de plomo[14] y se examinaron con el microscopio electrónico Carl Zeiss EM9. Por su parte, las mitocondrias se trataron en forma semejante, inmediatamente después de ser obtenidas por centrifugación diferencial.

RESULTADOS

PRIMERA SERIE. Mitocondrias aisladas del tejido cardíaco 12 horas después de la oclusión experimental de la arteria coronaria izquierda descendente.[1]

Perros sin infarto experimental

Las preparaciones de mitocondrias hechas de corazones de perros sometidos a todas las condiciones experimentales excepto que no se ligó la rama coronaria, mostraron índices de control respiratorio de 2.6 a 6.4 (4.9 ± 0.3) (promedio ± error estándar)

con beta-hidroxibutirato como substrato y de 1.6 a 5.2 (3.3±0.6) con succinato. Las relaciones P/O medidas como glucosa-6-fosfato/oxígeno, fueron de 1.43 a 2.88 (1.92 ±0.10) con beta-hidroxibutirato y de 1.12 a 1.99 (1.48±0.14) con succinato.

Perros con infarto experimental

Las preparaciones mitocondriales fueron tanto del tejido con infarto como del tejido aparentemente normal del mismo corazón.

Mitocondrias del tejido de control. Los índices del control respiratorio fueron de 1.9 a 4.6 (3.3±0.8), de 2.1 a 4.6 (3.3±0.7) y de 1.0±0.0, con beta-hidroxibutirato, succinato y dinucleótido reducido de nicotinamida adenina como substratos, respectivamente. Las relaciones glucosa-6-fosfato/oxígeno variaron de 1.45 a 2.02 (1.77±0.17), de 1.45 a 1.67 (1.59±0.07) y de 0.75 a 1.28 (0.99 ±0.16) para cada uno de los substratos, en el orden mencionado.

Mitocondrias del tejido con infarto. Los índices del control respiratorio no fueron mayores de 1 y no hubo síntesis de ATP durante la oxidación de los substratos a juzgar por la relación P/O que resultó de cero.

La figura 1 muestra los trazos electrocardiográficos de la pared anterior del ventrículo izquierdo a los 30 minutos y a las 12 horas de la oclusión arterial, respectiva-

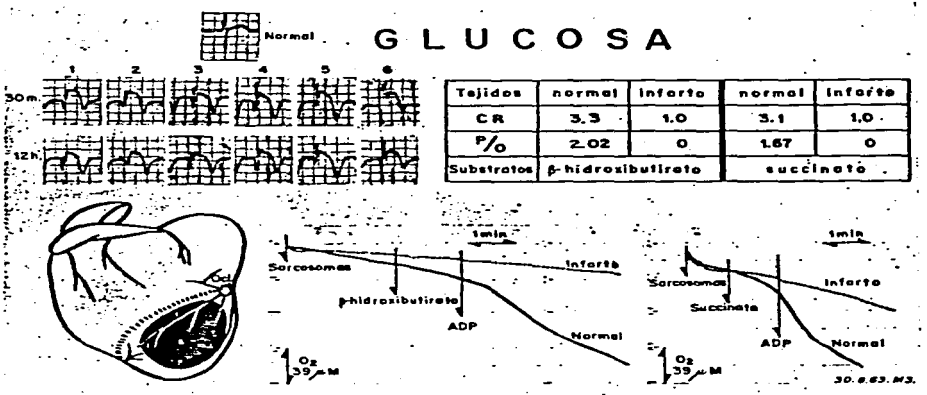

FIG. 1. Trazos electrocardiográficos tomados en los puntos marcados 1 a 6 de la pared anterior del ventrículo izquierdo del perro, a los 30 minutos y a las 12 horas de la oclusión de la rama coronaria izquierda descendente. Las gráficas de la parte inferior se obtuvieron con el electrodo de Clark durante la incubación respectiva de mitocondrias de la zona normal (línea continua) y de la zona con infarto (línea de puntos y rayas) con beta-hidroxibutirato (trazos de la izquierda) y con succinato (trazos de la derecha). Las flechas indican las adiciones. En la tabla se muestran los valores del índice del control respiratorio (CR) y de la relación glucosa-6-fosfato/oxígeno (P/O), con uno y otro substrato.

miente. En los registros se notan signos de lesión, de necrosis y de isquemia (segmento RS-T elevado, Q profunda y T negativa, respectivamente). Los trazos del consumo de oxígeno se obtuvieron con el electrodo de Clark acoplado a un registrador, durante la incubación de 0.2 ml de la preparación mitocondrial (2 a 5 mg de proteínas) en 3 ml de medio de reacción (manitol, sacarosa, tris(hidroximetil)aminometano, KCl y fosfatos de sodio, pH 7.2) y con 5 micromoles de DL-beta-hidroxibutirato (trazos de la izquierda) o de succinato (trazos de la derecha). La velocidad del consumo de oxígeno aumentó notablemente en las mitocondrias del tejido de control al agregar el substrato y sobre todo el ADP y no hubo respuesta en el caso de las mitocondrias aisladas del tejido con infarto.

SERIES POSTERIORES. Mitocondrias preparadas del corazón, 30 minutos, 4, 8 y 12 horas después de la ligadura de la arteria coronaria izquierda descendente.[2]

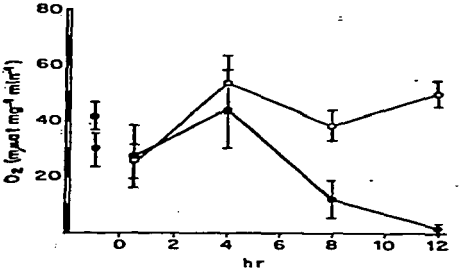

Fig. 2.- Promedio de las velocidades de consumo de oxígeno de mitocondrias de corazón de perro preparadas a diferentes tiempos después de la oclusión de la arteria coronaria descendente: área control ○ y área con infarto ●. También están incluidas las velocidades para mitocondrias de animales electrocutados ◐ y con la operación quirúrgica simulada ◒. El sustrato en la mezcla de incubación fue beta-hidroxibutirato.

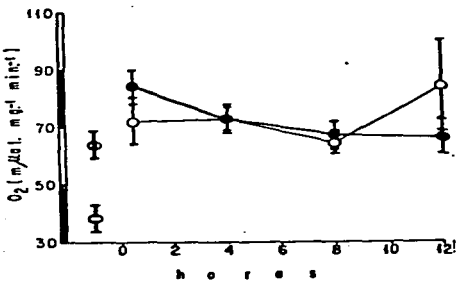

FIG. 3. Promedio de las velocidades de consumo de oxígeno de mitocondrias de corazón de perro. Las mismas condiciones que en la figura 2, excepto que el substrato fue succinato.

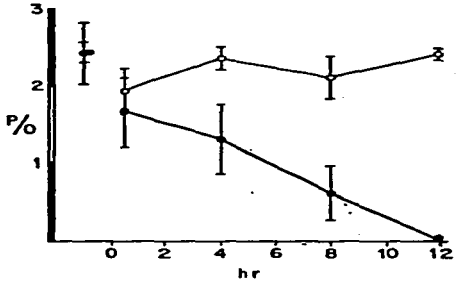

FIG. 4. Promedio de las relaciones P/O de mitocondrias de corazón de perro con beta-hidroxibutirato como substrato. Los símbolos corresponden a aquéllos de la figura 2.

Perros con infarto experimental

Mitocondrias del tejido de control. La velocidad promedio en el consumo de oxígeno de las mitocondrias del tejido de control de diferentes corazones a lo largo de 12 horas después de la ligadura arterial,

varió de 26 a 52 milimicroátomos de oxígeno por minuto por miligramo de proteína, cuando el beta- hidroxibutirato fue el substrato (Fig. 2) y de 64 a 84 con el succinato (Fig. 3). El promedio de las relaciones P/O

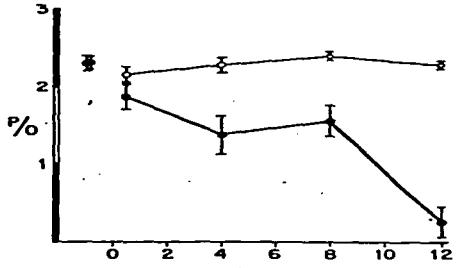

FIG. 5. Promedio de las relaciones P/O de mitocondrias de corazón de perro con succinato como substrato. Los símbolos corresponden a aquéllos de la figura 2.

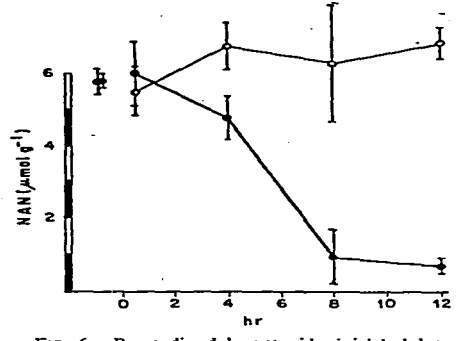

FIG. 6. Promedio del contenido inicial del total de nucleótidos de nicotinamida adenina en mitocondrias de corazón de perro. Los símbolos corresponden a aquéllos de la figura 2.

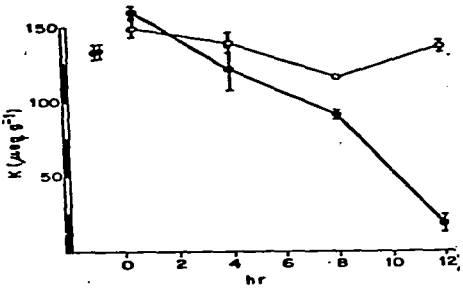

FIG. 7. Promedio del contenido de potasio en mitocondrias de corazón de perro. Los símbolos corresponden a aquéllos de la figura 2.

se mantuvo entre 1.92 y 2.41 con el beta-hidroxibutirato (Fig. 4) y entre 2.16 y 2.38 con el succinato (Fig. 5). El contenido inicial del total de nucleótidos de nicotinamida adenina en las mitocondrias fue de 5.50 a 6.86 micromoles por gramo de proteínas (Fig. 6) y disminuyó ligeramente en el período de equilibrio de 10 minutos y también después de 20 minutos de incubación a 30° en el aparato de Warburg. Los promedios del potasio contenido en las preparaciones mitocondriales fueron de 117 a 155 microequivalentes por gramo de proteína (Figura 7).

Mitocondrias del tejido con infarto. Las mitocondrias preparadas del tejido con infarto e incubadas con beta-hidroxibutirato (Fig. 2) consumieron menos oxígeno que las del tejido de control desde la octava hora de la oclusión arterial y prácticamente nada a las 12 horas. En contraste, cuando las mitocondrias de las mismas preparaciones se incubaron con succinato la velocidad del consumo de oxígeno se mantuvo la misma en todas las muestras (Fig. 3). Por lo que se refiere a las relaciones P/O, los valores obtenidos fueron bajos desde los 30 minutos después de la oclusión tanto con uno como

con otro substrato; sin embargo, en el caso del hidroxibutirato el descenso fue progresivo en las muestras posteriores (Fig. 4), mientras que con el succinato las relaciones durante las primeras ocho horas se mantuvieron semejantes a las medidas a los 30 minutos (Fig. 5); al final, en ambos casos, no hubo fosforilación en las preparaciones de las 12 horas. La cantidad de nucleótidos totales de nicotinamida adenina (Fig. 6) en las mitocondrias del tejido con infarto fue menor que en las del tejido de control a las 4 horas y bajó notablemente en las muestras de las 8 y de las 12 horas. Además, las mitocondrias del miocardio con infarto durante la incubación en el aparato de Warburg perdieron un porcentaje más alto de estos nucleótidos que las preparadas del tejido de control y esta pérdida fue mayor en los infartos de más larga duración.[2] El potasio mitocondrial (Fig. 7) disminuyó en las preparaciones de las 8 horas y todavía más a las 12 horas.

Cambios ultraestructurales de las células del miocardio

Tejido de control. La ultraestructura de las células del tejido cardíaco normal fue muy similar en las preparaciones obtenidas de diferentes perros a los diversos tiempos después de la ligadura de la rama coronaria. Los datos más relevantes se mencionan a continuación. Las miofibrillas en su mayoría se encontraron semicontraídas con desaparición virtual de las bandas I. Los espacios intermiofibrilares estaban ocupados por mitocondrias y elementos del sistema sarcotubular. Las mitocondrias en los espacios intermiofibrilares eran alargadas y de longitud más o menos semejante a la del sarcómero; las que ocupaban los espacios paranucleares y subsarcolemales también eran numerosas y de muy diversa forma y tamaño. Estos organelos mostraban su matriz moderadamente densa, conteniendo algunos gránulos y sus crestas se observaban orientadas paralelamente entre sí. Abundaban gránulos de glucógeno en las miofibrillas, en los espacios intermiofibrilares y sobre todo alrededor de las mitocondrias (Fig. 8).

Tejido con infarto. En cada caso se procuró tomar la muestra con infarto de la región que mostraba cambios electrocadiográficos peculiares de esta lesión; sin embargo, las alteraciones ultraestructurales observadas no fueron siempre semejantes entre sí en los perros correspondientes a tiempos iguales después de la oclusión arterial ni tampoco se notó que los daños fuesen progresivamente mayores a lo largo del tiempo. En otras palabras, hubo preparaciones del tejido infartado cuya apariencia era similar a la del tejido normal y cuyos valores P/O fueron altos, tanto en las muestras de 30 minutos como en las de los tiempos posteriores y hubo otras con valores P/O bajos y aun de cero, que mostraron notables alteraciones intracelulares semejantes entre sí independientemente del tiempo transcurrido. Por esto no es fácil en nuestra serie describir alteraciones características de cada uno de los tiempos escogidos. En general, las alteraciones intracelulares observadas fueron las siguientes: desgarramiento longitudinal y transversal de las miofibrillas; dilatación de los espacios intermiofibrilares y desaparición de los gránulos de glucógeno; hinchamiento de las mitocondrias, pérdida de la densidad de su matriz, ausencia de los gránulos intramitocondriales y desorganización y fragmentación de las crestas. Llamó la atención la presencia en el interior de algunos de estos organelos de grupos de crestas agrupadas en haces con una aparente conservación de su arquitectura y en otros la presencia de uno o dos grumos densos, así como también el vaciamiento parcial o total de algunas mitocondrias (Fig. 9).

Cambios ultraestructurales de las mitocondrias aisladas

Las mitocondrias de nuestras preparaciones no eran en su totalidad semejantes entre sí en cuanto a su apariencia, sobre todo

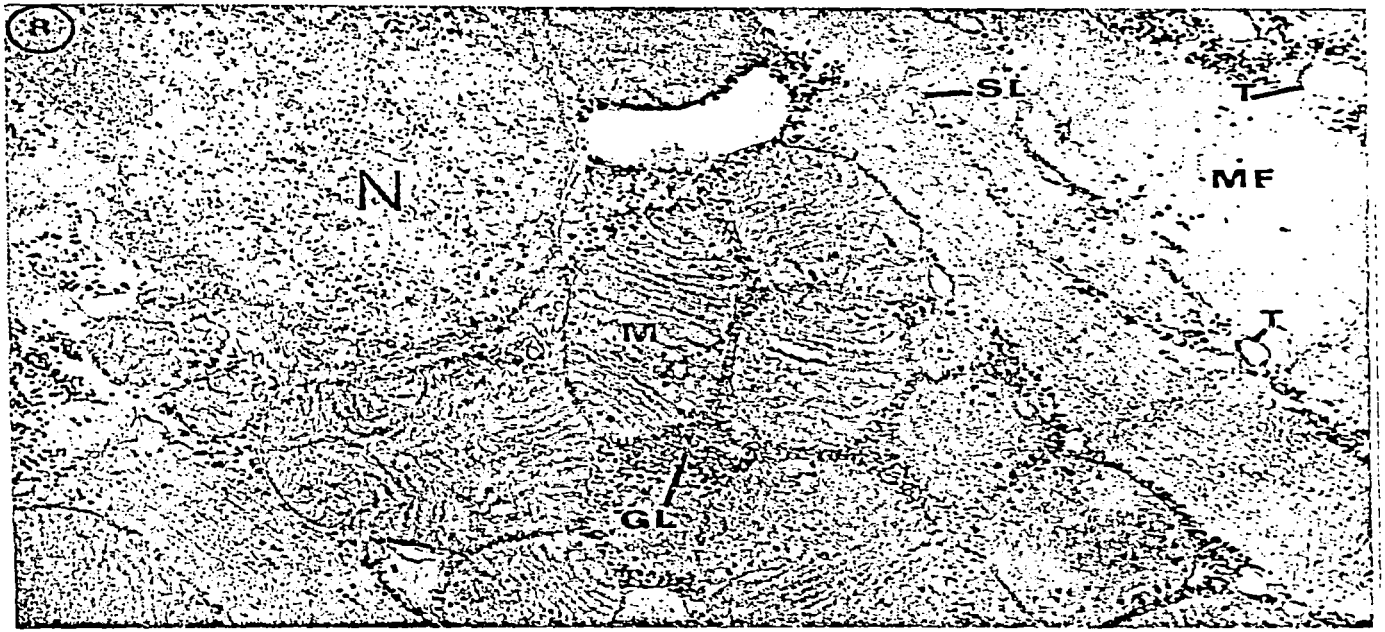

FIG. 8. Aspecto al microscopio electrónico de una porción de célula cardiaca del miocardio ventricular normal del perro. Se observa una parte del núcleo (N), abundantes mitocondrias (M) en la región parinuclear con sus crestas paralelas entre sí y con gránulos intramitocondriales, numerosos gránulos de glucógeno (GL), porciones de varias miofibrillas (MF) y elementos tanto del sistema sarcotubular longitudinal (SL) como del transversal (T). × 17,500.

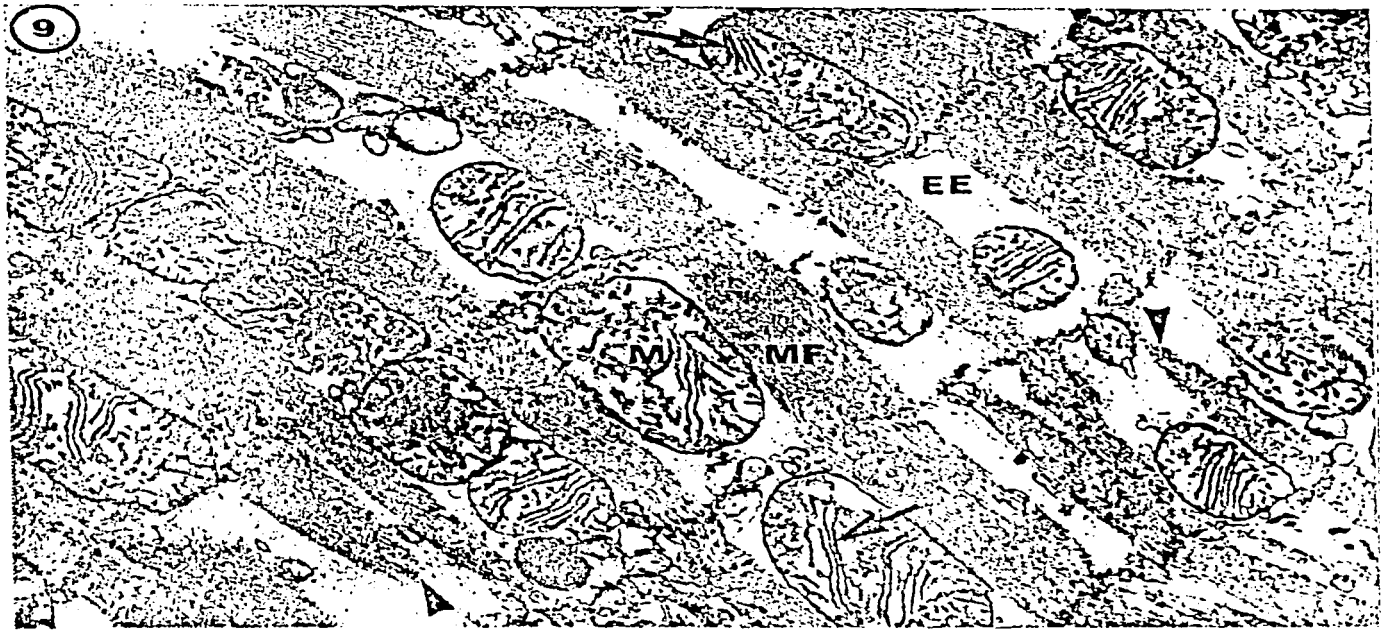

FIG. 9. Aspecto al microscopio electrónico de una porción de célula cardiaca del miocardio ventricular del área con infarto, a las doce horas de la ligadura de la rama coronaria descendente anterior en el perro. Se observa desgarramiento de las miofibrillas (cabezas de flecha) y amplios espacios entre ellas (EE); las mitocondrias muestran sus crestas fragmentadas (M) y en algunas se destaca la tendencia de las crestas a agruparse en haces (flechas); no se ven granulaciones citoplasmáticas ni intramitocondriales. × 17,500.

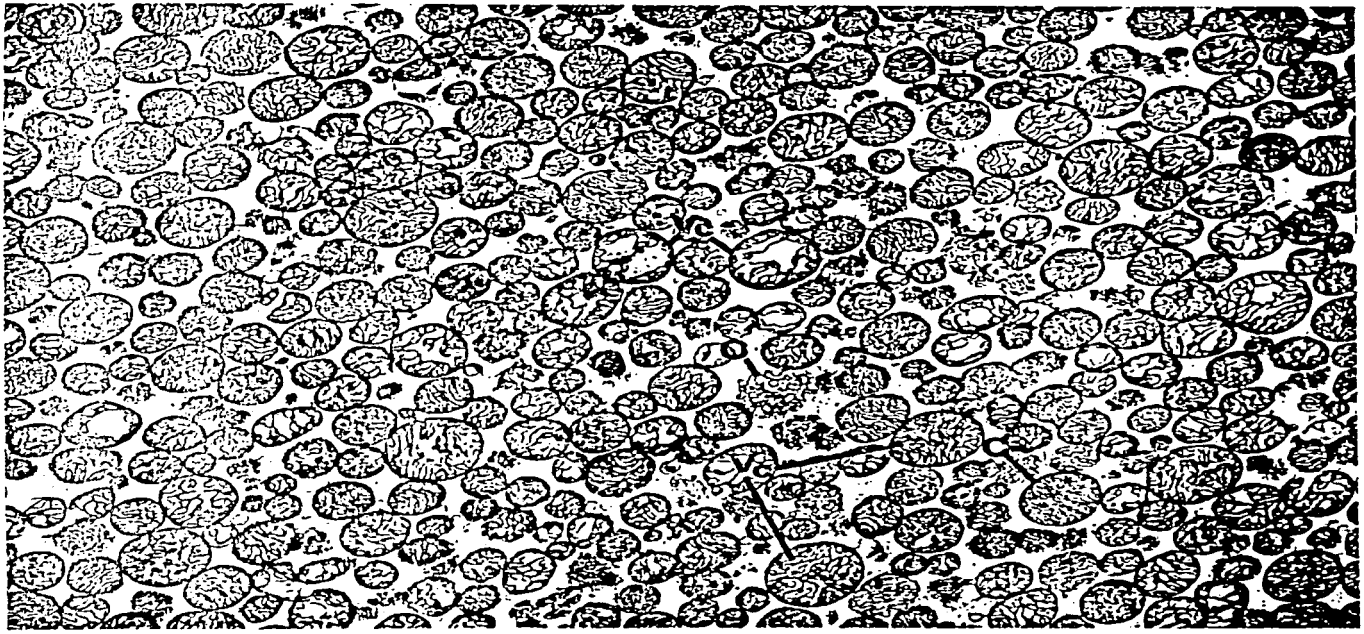

FIG. 10. Aspecto al microscopio electrónico de las mitocondrias separadas del tejido cardiaco normal del perro. Se observan mitocondrias condensadas (C) y en ovillo (O) así como vacuoladas (Vc), que son las que predominan. × 7,000.

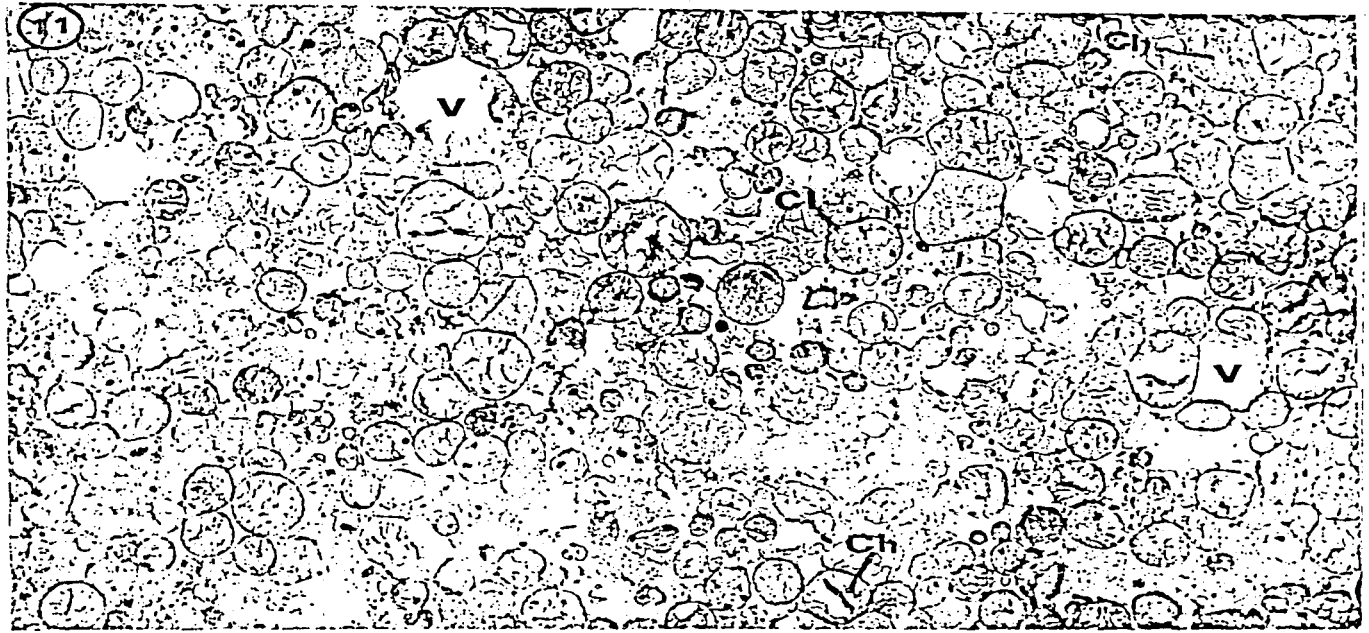

FIG. 11. Aspecto al microscopio electrónico de las mitocondrias del miocardio ventricular del área con infarto, después de doce horas de la oclusión de la rama coronaria en el perro. Son notables las alteraciones de estos organelos y la heterogeneidad de su apariencia. Se aprecian mitocondrias vacías (V), con cristolisis (Cl) y con crestas agrupadas en haces (Ch). × 7,000.

aquéllas del tejido con infarto, por lo que fue necesario clasificarlas arbitrariamente en los siguientes tipos:

Mitocondrias condensadas: las que tenían las crestas muy cercanas entre sí de manera que la cámara interna era casi imperceptible (C en la Fig. 10).

Mitocondrias en ovillo: aquéllas en que que las anteriores y con sus crestas separadas por estrechos espacios claros (Vc en la Figura 10).

Mitocondrias en ovillo: aquéllos en que sus crestas aparecían dispuestas en ovillo y no tenían membrana externa o bien ésta se encontraba a su alrededor claramente separada (O en la Figura 10).

Mitocondrias con cristolisis: las que aparecían llenas de un material relativamente denso a los electrones, tal vez formado por fragmentos de crestas (Cl en la figura 11).

Mitocondrias con haces de crestas: aquéllas que contenían uno o dos manojos muy peculiares de crestas (Ch en la Figura 11).

Mitocondrias vacías: verdaderas vesículas sin más estructura que algunos restos de crestas (V en la Figura 11).

Mitocondrias del tejido normal. Las preparaciones de mitocondrias del tejido normal (Fig. 10) fueron muy parecidas en todos los perros y la distribución porcentual de los diversos tipos mitocondriales (Tabla 1), en números redondos, resultó como sigue: vacuoladas 80, tipo en ovillo 10, condensadas 5, con cristolisis 2 y vacías 2. No se encontraron mitocondrias que tuviesen en su interior haces de crestas. Estas preparaciones del tejido normal con beta-hidroxibutirato como substrato mostraron un consumo de oxígeno de alrededor de 50 milimicroátomos por minuto y por miligramo de proteína y relaciones P/O del orden de 2.5.

Mitocondrias del tejido con infarto. La distribución de los tipos mitocondriales así como los valores del consumo de oxígeno y de la relación P/O a los 30 minutos después de la oclusión arterial resultaron semejantes a los encontrados en el tejido normal (Tabla 2). Con relación a las preparaciones hechas a las 4, 8 y 12 horas, respectivamente, sólo se incluyen en la Tabla 2 los datos morfológicos de aquéllas con valores P/O menores de 1.5 y se descartan las que mostraron cifras cercanas a 2.5 ya que en estas últimas la distribución porcentual de los tipos mitocondriales fue parecida a la de las las preparaciones del tejido normal. Así, a las 4 horas, en uno de cuatro experimen-

TABLA 1

VALORES PROMEDIO DE LA DISTRIBUCION PORCENTUAL DE LOS DIFERENTES TIPOS DE MITOCONDRIAS ENCONTRADOS EN LAS PREPARACIONES DEL TEJIDO MIOCARDICO NORMAL

Tipos	*Símbolos*	½	4	8	12
		(horas después de la oclusión arterial)			
Condensadas	C	8	2	3	5
Vacuoladas	Vc	81	73	84	85
En ovillo	O	10	15	9	8
Con cristolisis	Cl	1	4	2	1
Con haces de crestas	Ch	0	0	0	0
Vacías	V	0	6	2	1
Muestras estudiadas		3	4	5	4
Muestras incluidas en el promedio		3	4	5	4

Ver figura 10.

TABLA 2

VALORES PROMEDIO DE LA DISTRIBUCION PORCENTUAL DE LOS DIFERENTES TIPOS DE MITOCONDRIAS ENCONTRADOS EN LAS PREPARACIONES DEL TEJIDO MIOCARDICO CON INFARTO

Tipos	*Símbolos*	*½*	*4*	*8*	*12*
		(horas después de la oclusión arterial)			
Condensadas	C	4	0	1	0
Vacuoladas	Vc	85	54	36	40
En ovillo	O	9	11	2	4
Con cristolisis	Cl	1	22	27	23
Con haces de crestas	Ch	0	0	25	16
Vacías	V	1	13	9	17
Muestras estudiadas		4	1	3	3
Muestras incluidas en el promedio*		4	4	5	4

* Con excepción del grupo de ½ hora, en los demás grupos sólo se incluyeron las muestras mitocondriales cuyo índice P/O fue menor de 1.5.
Ver figura 11.

tos, el porcentaje de mitocondrias vacuoladas fue menor que en el tejido sin daño, aumentaron las que mostraban cristolisis y las vacías y aparecieron las que tenían en su interior crestas agrupadas en haces siendo la relación P/O de 1.5. A las ocho horas, en tres de cinco experimentos, se vieron todavía menos mitocondrias vacuoladas y mayor número de las otras formas. Estas preparaciones tuvieron relaciones P/O cercanas a cero y una velocidad de consumo de oxígeno menor de 19. Finalmente, en tres de cuatro experimentos hechos a las doce horas de la ligadura, el consumo de oxígeno fue cero, el porcentaje de mitocondrias destruidas aumentó y las pocas formas reconocibles mostraron cambios como las de las ocho horas.

DISCUSION

Las mitocondrias del corazón normal del perro mostraron, con la adenosina difosfato como aceptor, índices de control respiratorio de 1.9 a 6.4 con beta-hidroxibutirato y de 1.6 a 5.2 con succinato, medidos con el electrodo de oxígeno. Estas cifras se parecen a las obtenidas también en el polarógrafo con mitocondrias de corazón de buey incubadas con piruvato[16] y si se toman como criterio para apreciar la conservación de la estructura mitocondrial,[9] significan que las mitocondrias del tejido normal se obtuvieron satisfactoriamente preservadas. El hecho de que los valores de la fosforilación oxidativa con el succinato resultaron semejantes a los observados con hidroxibutirato cuando se usó la técnica manométrica y se midió el fosfato inorgánico, se debe a que los valores P/O medidos en esas condiciones revelan la fosforilación que ocurre no solo a nivel del succinato sino también a nivel de los substratos subsecuentes que se forman a través del ciclo de Krebs.[7] En cuanto al dinucleótido reducido de nicotinamida adenina es probable que no haya sido oxidado a través de la cadena respiratoria, pues la adición de ADP no estimuló el consumo de oxígeno y el valor P/O, que resultó cercano a la unidad, fue menor que el que se encontró cuando solo se oxidaban los substratos endógenos.[1] Lehninger[8] ha sugerido que este dinucleótido reducido es oxidado a través de un sistema mitocondrial externo no acoplado a la fosforilación y que solamente tiene acceso al sistema interno cuando se altera la permeabilidad mitocondrial, cosa que no parece que ocurra en nuestras preparaciones del

tejido normal en vista de los bajos valores P/O obtenidos con este substrato. Por lo que se refiere a la velocidad de consumo de oxígeno de las mitocondrias en presencia de ADP y de beta-hidroxibutirato o de succinato, la respiración fue mayor, casi el doble, con este último compuesto. Strickland[19] encontró una relación semejante entre ambos substratos con mitocondrias de hígado de rata y Smith[16] obtuvo, con mitocondrias de corazón de buey incubadas con succinato, cifras que se parecen a las mostradas por las mitocondrias del corazón del perro. La diferencia en la velocidad del consumo de oxígeno depende de la actividad del complejo enzimático responsable de la oxidación del substrato correspondiente[19] y también de la permeabilidad particular de las mitocondrias para cada substrato.[9]

No hubo diferencias entre las mitocondrias preparadas de la pared posterior del ventrículo izquierdo ya fuesen de corazones en los que se ligó la rama coronaria o de aquéllos sin ligadura arterial.

En cuanto a las mitocondrias aisladas del tejido con infarto, algunas de las alteraciones bioquímicas estudiadas se empezaron a manifestar desde los treinta minutos de la ligadura de la arteria (valores bajos de P/O), otras aparecieron a las cuatro horas (disminución del contenido de nucleótidos de nicotinamida adenina) y las demás a las ocho (bajo consumo de oxígeno con beta-hidroxibutirato y pérdida del ion potasio) y a las doce horas (pérdida del control respiratorio); sin embargo, ninguno de los valores estudiados se modificó significativamente tan temprano como los trazos electrocardiográficos.[17] Es interesante observar que el sistema de oxidación del succinato se mantuvo funcionalmente activo al menos hasta doce horas después de la oclusión arterial que fue el tiempo máximo de estudio y en cambio el sistema que participa en la oxidación del beta-hidroxibutirato se deterioró desde la octava hora. Si se acepta que la cadena respiratoria es común en su parte final para ambos tipos de substratos, estos hallazgos significan que los componentes del complejo I,[6] o las deshidrogenasas asociadas al NAD o el contenido de NAD y NADH, se pierden o se inactivan, unos o todos, a la vez o separadamente, en tanto que los complejos II, III y IV[6] y las concentraciones de coenzima Q y de citocromo c se mantienen suficientes desde el punto de vista funcional. El hecho de que los valores de la fosforilación se hubiesen visto más bajos desde el principio del infarto cuando se midieron con hidroxibutirato como substrato y se hubiesen mantenido normales en las primeras ocho horas cuando se estudiaron con succinato, posiblemente significa que el deterioro se inicia en el primer sitio de fosforilación o sea entre el NAD y la flavoproteína, mientras se conservan activos hasta la octava hora el segundo y el tercero, esto es, aquéllos entre el citocromo b y el c y entre el citocromo c y el oxígeno. Finalmente, a las doce horas, estos dos sitios también se dañaron a juzgar por la ausencia completa de fosforilación en las mitocondrias incubadas con uno u otro de los substratos mencionados. En el caso del hidroxibutirato, al deterioro del primer sitio de fosforilación se agregan los efectos del daño a nivel del complejo I de la cadena respiratoria, lo que explicaría el descenso aparentemente progresivo de la relación P/O que se ve en la figura 4 y que contrasta con la evolución de estos valores en presencia de succinato (Fig. 5). Por los datos obtenidos en el presente trabajo se puede postular que la alteración de la cadena respiratoria se debe principalmente a la pérdida de los nucleótidos de nicotinamida adenina de las mitocondrias de la zona del infarto, pérdida que aun se manifestó in vitro durante el periodo de incubación en el aparato de Warburg. Por último, la salida del ion potasio parece ser una consecuencia inmediata de la disminución de los procesos de fosforilación.[4]

Desde el punto de vista estructural, las mitocondrias preparadas del tejido normal

mostraron en su mayoría el aspecto correspondiente al tipo que hemos llamado vacuolado y tanto la relación P/O como el control respiratorio y la velocidad del consumo de oxígeno fueron semejantes a los valores encontrados por otros autores en otras especies animales.

En las mitocondrias del tejido con infarto las cifras del consumo de oxígeno y de la fosforilación oxidativa fueron bajas cuando las mitocondrias tenían las crestas fragmentadas. En cuanto a los haces de crestas que se vieron en el interior de algunas mitocondrias del tejido con infarto parece que eran restos de estas formaciones que no se habían fragmentado, aunque también pudiera tratarse de estructuras recién formadas en mitocondrias previamente desorganizadas. A medida que transcurrió el tiempo del infarto no solo se acentuó el grado de ruptura sino que aumentó el número de las mitocondrias dañadas. En el artículo de Ekholm y colaboradores,[3] publicado recientemente y que se refiere a los efectos de periodos muy cortos de anoxia, las preparaciones mitocondriales del corazón del perro dejan mucho que desear en cuanto a homogeneidad e integridad.

RESUMEN

Se produjo una zona de infarto en la pared anterior del corazón del perro al ligar la rama descendente de la arteria coronaria izquierda. Las mitocondrias se aislaron por centrifugación diferencial tanto del miocardio con infarto como del tejido normal, treinta minutos, cuatro, ocho y doce horas después de la oclusión de la coronaria. En estas preparaciones se determinó el control respiratorio, el consumo de oxígeno, la fosforilación oxidativa y la concentración de nucleótidos de nicotinamida y de potasio. Además se observaron al microscopio electrónico.

En el miocardio dañado la síntesis de adenosina trifosfato concomitante al consumo de oxígeno disminuyó desde los primeros minutos en las mitocondrias incubadas con hidroxibutirato y llegó a cero a la duodécima hora. Cuando se empleó como substrato el succinato la fosforilación oxidativa decayó solo hasta después de la octava hora. Se sugiere que desde los primeros minutos se daña el sitio de fosforilación entre los nucleótidos de nicotinamida y las flavoproteínas y más tarde los dos restantes.

Las mitocondrias aisladas ocho horas después de haberse provocado el infarto consumieron menos oxígeno que las preparadas del tejido normal con el substrato beta-hidroxibutirato y a las doce horas el consumo prácticamente fue cero. Las incubadas con succinato utilizaron una cantidad de oxígeno similar a las del miocardio normal en todas las mediciones hechas en el curso de las doce horas del experimento. Se sugiere que la parte inicial de la cadena respiratoria del beta-hidroxibutirato se empieza a deteriorar a partir de la oclusión arterial y en cambio se conserva activa la porción de la cadena que es común a la del succinato.

En las preparaciones mitocondriales correspondientes a las doce horas de la oclusión arterial no hubo control respiratorio independientemente del substrato utilizado, lo que indica una gran alteración de la estructura mitocondrial.

La concentración de nucleótidos de nicotinamida adenina en las mitocondrias del miocardio con infarto bajó ligeramente a la cuarta y notablemente a la octava hora, lo cual explica el deterioro observado en los sistemas de oxidación en los que participa esta coenzima, como es la cadena respiratoria del beta-hidroxibutirato, y la conservación de los sistemas en los que no están involucrados estos nucleótidos, como es el caso del succinato.

La concentración del potasio en las mitocondrias disminuyó desde la cuarta hora y alcanzó el nivel más bajo entre la octava y la duodécima hora probablemente a consecuencia de la interrupción de los procesos de fosforilación oxidativa.

Las alteraciones ultraestructurales observadas en la célula cardiaca fueron la dilata-

ción de los espacios intermiofibrilares, la desaparición de los gránulos de glucógeno, la ruptura de las miofibrillas y el hinchamiento de las mitocondrias con desorganización y fragmentación de sus crestas y la pérdida de sus gránulos así como la aparición de grupos de crestas agrupadas en haces.

En las preparaciones de mitocondrias aisladas del tejido normal predominó el tipo vacuolado. Las formas con cristolisis y las vacías abundaron en las preparaciones del tejido con infarto y se vieron además en éstas las que mostraban haces de crestas.

En conclusión, las funciones mitocondriales estudiadas se encontraron deterioradas al tiempo que había una casi completa desorganización de las crestas de estos organelos, lo cual ocurrió sobre todo a las ocho y a las doce horas después de la oclusión experimental de la arteria coronaria en el perro.

SUMMARY

An infarcted area was produced on the anterior wall of the dog heart by the ligature of the left descending coronary artery. Mitochondria were isolated by differential centrifugation from both the infarcted and normal myocardium at thirty minutes, four, eight and twelve hours after the arterial occlusion. Respiratory control, oxygen consumption, oxidative phosphorylation, nicotinamide adenine nucleotides and potassium content were determined in these preparations. Electron microscopic observations were also performed.

The adenosine triphosphate synthesis coupled to oxygen consumption fell after the first minutes of infarction in the mitochondria incubated with hydroxybutyrate and reached zero at the twelfth hour; however, with succinate the oxidative phosphorylation fall occurred only after the eighth hour of the arterial occlusion. It is suggested that the phosphorylation site between nicotinamide nucleotides and flavoproteins becomes damaged since the first minutes and later on the two others are also involved.

Mitochondria isolated eight hours after the production of the infarct consumed less oxygen with the substrate beta-hydroxybutyrate than those prepared from the normal tissue and at the twelfth hour the consumption was zero. With succinate the oxygen consumption rate was similar in mitochondria prepared from the normal and from the infarcted tissues during the twelve hours of the experiment. It is suggested that the initial portion of the respiratory chain linked to the oxidation of beta-hydroxybutyrate begins to be damaged at the time the artery is occluded while that segment of the chain common to the system linked to the oxidation of succinate remains active.

No respiratory control was observed with mitochondria prepared twelve hours after the infarction irrespectively of the substrate used meaning that profound structural mitochondrial alterations had occurred.

The concentration of the total nicotinamide adenine nucleotides in mitochondria from the infarcted tissue decreased slightly at the fourth hour and remarkably at the eighth hour and this could explain the failure of the oxidative systems which need this coenzyme such as that for the beta-hydroxybutyrate, and the preservation of those systems where these nucleotides are not involved like the one for succinate.

The potassium concentration diminished since the fourth hour and reached the lowest level between the eighth and the twelfth hours, probably as a consequence of the decay of the oxidative phosphorylation processes.

The ultrastructural alterations observed in the cardiac cell were dilatation of intermyofibrillar spaces, disappearance of glycogen granules, rupture of myofibrils, mitochondrial swelling with disorganization and fragmentation of their cristae, lost of their granules and appearance of bundles of cristae.

The vacuolated type of mitochondria pre-

dominated in preparations from normal tissues. Mitochondria with cristolysis and those of the empty type were abundant in preparations from the infarcted area together with mitochondria showing bundles of cristae.

In conclusion, the mitochondrial functions were found impaired at the time in which there was an almost complete rupture of the cristae of these organelles and this occurred namely eight and twelve hours after the experimental occlusion of the coronary artery in the dog.

RÉSUMÉ

Un infarctus antérieur fut produit dans le coeur du chien, par ligature de l'artère coronaire descendante gauche. Les mitochondries furent isolées par centrifugation différentielle du myocarde infarci et du tissu normal, trente minutes, quatre, huit et douze heures après l'occlusion de la coronaire. Dans ces préparations, on détermina le contrôle respiratoire, la consommation d'oxygène, la phosphorylation oxydative et la concentration du potassium et des nucléotides de nicotinamide adenine. Par ailleurs, on examina ces préparations au microscope électronique.

La synthèse de l'adénosine triphosphate, liée à l'utilisation d'oxygène, diminua dès les premières minutes de l'installation de l'infarctus dans le mitochondries incubées avec de l'hydroxybutyrate. Ce fait est dû probablement à des altérations précoces de la phosphorylation associée au passage d'électrons entre les nucléotides de la nicotinamide et les flavoprotéines. Quant on employa comme substrat du succinate, la phosphorylation oxydative continua jusqu'à la huitième heure. La synthèse de l'ATP tomba a zéro vers la duozième heure avec les deux substrats. Ces alterations tardives sont probablement dues à l'atteinte des autres niveaux de la formation d'ATP.

Les mitochondries du myocarde atteint, isolées huit heures aprés l'installation de l'infarctus ou plus tard, consommèrent moins d'oxygène avec le substrat beta-hidroxybutyrate, que celles prélevées du tissu normal. En revanche, dans tous les échantillons préparés au cours de douze heures d'observation en employant comme substrat du succinate, les mitochondries du tissu atteint consomèrent la même quantité d'oxygène que celles du myocarde normal. La consommation d'oxygène n'augmentait pas dans les mitochondries prélevées douze heures aprés avoir produit l'infarctus, quand on y ajoutait de l'adénosine diphosphate, indépendamment du substrat employé. Par conséquent, dans ces cas, le contrôle respiratoire était nul.

Le contenu des nucléotides de la nicotinamide adénine, dans les mitochondries du myocarde infarci, diminua légèrement à la quatrième heure et, en mesure considérable, à la huitième. Ceci pourrait expliquer la détérioration des systèmes d'oxydation qui utilisent ces coenzymes, telle la voie suivie par le beta-hydroxybutyrate. Par contre,l'activité des systèmes où ces nucléotides n'interviennent pas, par exemple, la voie d'oxydation du succinate, était bien conservée.

La concentration de potassium intramitochondrial diminua quatre heures après l'installation de l'infarctus et atteignit le niveau le plus bas entre la huitième heure et la douzième. Cela s'explique probablement par l'interruption des processus de la phosphorylation oxydative.

Au microscope électronique, on observa notamment dilatation des espaces interfibrillaires, la disparition des granules du glycogène, la rupture des miofibrilles, le gonflement des mitochondries avec desorganization et fragmentation de ses crêtes, la disparition des granules mitochondriales et la présence de crêtes, la disparition des granules mitochondriales et la présence de crêtes groupées en faisceaux.

Dans les préparations de mitochondries isolées du tissu normal, il y avait surtout des formes mitochondriales de type vacuolaire. En revanche, dans les préparations du tissu atteint, on vit apparaître des éléments montrant des crêtes en faisceaux et on remarqua

l'apparition de mitochondries avec cristolyse et de ceux dits vides.

En conclusion, cette recherche expérimentale montra une détérioration de certaines fonctions des mitochondries parallèle à la désorganisation presque complète des crêtes mitochondriales, particulièrement dans les préparations obtenues huit et douze heures après l'occlusion de l'artère coronaire antérieure gauche chez le chien.

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Nicotinamide coenzymes in heart and coronary blood during myocardial infarction

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NÚÑEZ, ROSARIO, EDMUNDO CALVA, ELISA BRIONES, AND FERNANDO LÓPEZ-SORIANO. *Nicotinamide coenzymes in heart and coronary blood during myocardial infarction.* Am. J. Physiol. 226(1):73–76. 1974.—Nicotinamide adenine dinucleotides were measured in normal and infarcted dog cardiac tissue and in arterial and coronary sinus blood. Myocardial infarction was produced in the anterior wall of the left ventricle by ligating the descending coronary artery. The damage was evaluated by epicardial electrocardiograms. Total dinucleotides in normal cardiac tissue (nmol/g wet wt) were NAD 676, $NADH_2$ 276, NADP 43, and $NADPH_2$ 32, and in arterial and coronary sinus blood (μmol/kg) NAD 35 and $NADH_2$ 24. Concentrations of the four coenzymes were lower and the $NADH_2$/NAD ratio was higher in infarcted than in noninfarcted tissue. No statistically significant changes were observed in the level and the ratio of these dinucleotides in the coronary sinus blood after occlusion of the artery. The steady loss from the myocardial infarcted portion may be due to a leakage toward the bloodstream with subsequent hydrolysis there and/or to the action of a NAD glycohydrolase whose activity is disclosed in the damaged tissue. Blood contains a similar hydrolase which splits added dinucleotides.

nicotinamide adenine dinucleotides; NAD glycohydrolase; myocardial ischemia; dog cardiac tissue

THE BLOCKADE of the respiratory chain detected in infarcted myocardial tissue soon after the ligature of the coronary artery seems to be related to the loss of mitochondrial nicotinamide adenine dinucleotides (6, 7). Govier (9) reported a low coenzyme I content in samples obtained 2 h after coronary artery occlusion. In the present work all of the nicotinamide adenine dinucleotides (NAD, $NADH_2$, NADP, and $NADPH_2$) were measured separately in cardiac tissue of dogs with acute myocardial infarction. Whole tissue was analyzed instead of separated fractions because the distribution of the nucleotides is liable to undergo very rapid changes during the process of tissue fractionation (10). Owing to the possibility that the loss of these coenzymes might be a result of their leakage into the bloodstream, NAD and $NADH_2$ were also determined in blood samples taken from the coronary sinus.

MATERIALS AND METHODS

Sixty-one mongrel dogs weighing 10–23 kg were divided into two groups: one for the study of levels of nicotinamide adenine dinucleotides in normal cardiac tissue (normal hearts) and the other for the study of the content of these nucleotides in myocardial tissue and in the coronary sinus blood during the experimentally produced cardiac infarction (infarcted hearts).

Normal hearts. The heart was excised from 26 dogs anesthetized with intravenous pentobarbital (30 mg/kg). In 16 of these animals the organ was immediately perfused with 40 ml of cold 0.05 M nicotinamide (Sigma N3376) in Ringer solution injected through both the descending and the circumflex branches of the left coronary artery. The remaining 10 hearts were perfused with plain Ringer solution. Four samples of the left ventricle, two from the anterior wall and two from the posterior, were taken from each heart. Manipulations of these samples and measurement procedures of NAD, $NADH_2$, NADP, and $NADPH_2$ were identical to those described below under cardiac tissue samples.

Infarcted hearts. Thirty-five dogs were anesthetized as described above, and after tracheal intubation positive-pressure ventilation was maintained with a Palmer pump using room air. The chest was opened via a left intercostal incision, and the left descending coronary artery was occluded near its origin in one step.

Unipolar epicardial electrograms were recorded by placing the exploring electrode on eight points of the anterior aspect of the left ventricle (5). The exploring electrode was a cotton pad wet in warm isotonic NaCl solution, attached to the tip of the lead, and stretched and twisted at its free end. One set of records was taken 30 min after the arterial occlusion and the other 5 min before removing the heart. The degree of the injury was estimated roughly by the magnitude of the RS-T segment displacement. Subendocardial and transmural necrosis were revealed by pathological Q waves (wide and slurred) and QS complexes, respectively.

Cardiac tissue samples. Twenty-seven of these 35 dogs were divided into four groups and sacrificed 30 min, and 2, 4, and 48 h, respectively, after the coronary artery ligation. A removable ligature was placed in the artery of the animals of the first three groups, and a permanent bond was put in those to be kept alive 48 h. In this group the chest was closed by suturing, and the animals were maintained anesthetized with sporadic intraperitoneal injections of pentobarbital (128 mg). All dogs received a continuous intravenous infusion of 10% glucose solution (1.5 ml/min).

Those areas on the left ventricle which showed the greatest electrophysiological damage were marked with colored pins. Immediately after the heart was removed, the coro-

nary ligature was loosened and the left ventricle was perfused with the nicotinamide solution. The time elapsed between removal and perfusion was less than 2 min. Hearts from dogs sacrificed 48 h after the coronary occlusion were not perfused. Two samples of damaged tissue (infarcted tissue) and two from the posterior wall near the ventricular base (noninfarcted tissue) were taken from each heart by using a 1.27-cm cork borer. Each sample consisted of approximately 500 mg of tissue wet weight.

Tissue extracts to measure free and bound nicotinamide adenine dinucleotides were prepared following the method of Lowry, Passonneau, Schulz, and Rock (11). One sample of infarcted and one of noninfarcted tissue were immersed immediately in separate cooled weighing bottles containing 4 ml of acid medium (0.01 M H_2SO_4 and 0.1 M Na_2SO_4). Two analogous samples were treated similarly with alkaline medium (0.02 N NaOH with 0.5 mM L-cysteine-HCl, freshly made). The tissue fragments were cut with scissors, and the flasks were weighed again to determine the exact amount of the sample. The time elapsed between the removal of the heart and the immersion of the tissues in the media was around 3 min. Finally, each specimen was suspended in 20 ml of medium. *n*-Hexane was added to the extracts before centrifugation (3).

The technique of Lowry, Roberts, and Kapphahn (12) was utilized in the measurement of the dinucleotides.

Coronary sinus blood samples. In the remaining eight dogs, one catheter was introduced into the femoral artery and another into the coronary sinus via the right auricle. The tip of the sinus catheter was placed at the outlet of the vena cordis magna (8), and the position of the catheter apex was verified manually during the experiment. Heparin (500 U/250 ml) in 10% glucose solution was continuously infused through the sinus catheter (60 ml/h). Arterial and coronary sinus blood control samples were withdrawn simultaneously. The first sample was taken before the arterial occlusion, and others were taken 2, 4, 9, and 23 h after the descending branch of the left coronary artery was occluded.

At least 10 ml of blood were allowed to drain by the catheters before specimens approximately equivalent to 250 mg were taken with 1 ml medical-type syringes fitted with Chaney adaptors (Hamilton Co.). Two blood samples from each catheter were immediately transferred into preweighed tubes containing 7.8 ml of alkaline medium (0.1 M Na_2CO_3 and 0.02 M $NaHCO_3$) and 9.3 ml of acid medium (0.01 M H_2SO_4 and 0.1 M Na_2SO_4), respectively (11). Less than 30 s elapsed between the removal of the blood samples and their mixture with the media. Once the blood had been added, the tubes were weighed again. Finally, the samples were brought to a 10-ml volume.

The NAD and $NADH_2$ in the blood extracts were measured by an enzymatic cycling method (11). Values are expressed in micromoles per kilogram of blood and on the basis of a hematocrit value of 45%, since the bulk of these dinucleotides is contained in the red cells (4).

RESULTS

Normal hearts. The mean value of the NAD contained in the tissue samples from normal hearts appeared higher when the left ventricle was perfused with the nicotinamide solution than when perfused only with Ringer. The values for the other dinucleotides did not change significantly (Table 1).

The average level of the total nicotinamide adenine dinucleotides in the anterior wall of the left ventricle from the dog heart was 1,007 ± 106 (nmol/g wet wt), which includes 676 ± 50 of NAD, 276 ± 36 of $NADH_2$, 43 ± 14 of NADP, and 32 ± 5 of $NADPH_2$. Similar values were found in samples taken from the posterior wall of the cardiac left ventricle (Table 2).

Infarcted hearts. The average concentrations of NAD, $NADH_2$, NADP, and $NADPH_2$ in the noninfarcted cardiac tissue were similar to those found in samples from normal hearts, except the NAD value in the 48-h sample, which was around 17% lower (Figs. 1, 2).

In the infarcted cardiac tissue taken 30 min after the ligature of the artery, the average levels of these dinucleotides were not different from those found in the noninfarcted samples; however, the content of each one of the four coenzymes decreased remarkably later on (Figs. 1, 2). The $NADH_2$/NAD ratio was found to have increased 2 h after the arterial occlusion.

In the coronary sinus blood removed before the ligature of the coronary artery, the concentrations of NAD and $NADH_2$ were the same as those found in the femoral artery blood. The NAD represented approximately 60% of the total nicotinamide coenzymes. After the arterial occlusion, no significant changes were observed in the level and the ratio of these dinucleotides in the blood taken from the

TABLE 1. *Effect of nicotinamide perfusion on nicotinamide adenine dinucleotide content of left ventricle anterior wall of the dog heart*

	Ringer Without Nicotinamide (n = 10)	Ringer With Nicotinamide (n = 16)	P Values
	nanomoles/g wet wt		
NAD	515 ± 62	661 ± 75	<0.001
$NADH_2$	250 ± 29	272 ± 33	<0.20
NADP	51 ± 16	45 ± 15	<0.40
$NADPH_2$	25 ± 14	32 ± 12	<0.30
NAD + NADP	531 ± 54	681 ± 106	<0.001
$NADH_2$ + $NADPH_2$	275 ± 31	300 ± 45	<0.10

Values are means ± SD; *n* = number of dogs.

TABLE 2. *Levels of nicotinamide adenine dinucleotides in the left ventricle of the normal dog heart perfused with nicotinamide immediately after its removal*

	Anterior Wall	Posterior Wall	P Values
NAD	676 ± 50	629 ± 80	<0.2
$NADH_2$	276 ± 36	264 ± 30	<0.4
NADP	43 ± 14	41 ± 10	>0.8
$NADPH_2$	32 ± 5	34 ± 11	<0.7
NAD + NADP	697 ± 84	654 ± 79	>0.9
$NADH_2$ + $NADPH_2$	310 ± 39	299 ± 24	<0.5
Total*	1,007 ± 106	953 ± 81	<0.2
$NADH_2$/NAD*	0.40 ± 0.04	0.41 ± 0.04	<0.5
$NADPH_2$/NADP*	0.81 ± 0.31	0.84 ± 0.31	>0.9

Values are means ± SD; number of dogs = 12. * Values calculated by adding those experimentally determined.

coronary sinus (Tables 3, 4), even though abnormal tracings were apparent in the epicardial electrograms.

The hematocrit in the animals remained almost the same during each one of all the experiments (±3 % of the initial value).

DISCUSSION

The nicotinamide added to the Ringer solution used to perfuse the heart probably inhibits (13) the NAD glycohydrolase activity present in dog cardiac tissue (unpublished data) and consequently prevents the fall (around 22 %) of NAD observed in hearts perfused only with Ringer. This interpretation probably explains the 17 % fall observed in the noninfarcted tissue taken from the nonperfused hearts of dogs sacrificed 48 h after the coronary artery ligature.

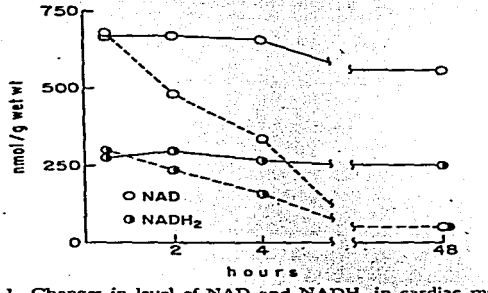

FIG. 1. Changes in level of NAD and $NADH_2$ in cardiac muscle from dogs with experimental myocardial infarction. Noninfarcted tissue ——. Infarcted tissue – – –. Every point represents average result of 8, 7, 7, and 5 animals studied at 1/2, 2, 4, and 48 h after infarction, respectively.

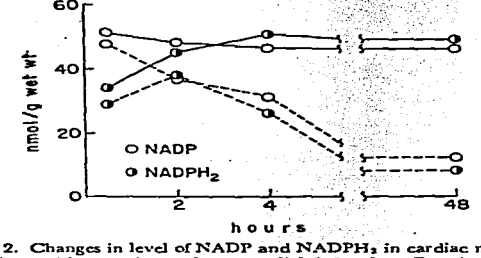

FIG. 2. Changes in level of NADP and $NADPH_2$ in cardiac muscle from dogs with experimental myocardial infarction. Description is same as for Fig. 1.

The figures published by Michal et al. (14) for NAD, $NADH_2$, and $NADPH_2$ in dog myocardial control tissue appear without the units in which they were measured; therefore, they cannot be used as a reference. Govier (9) reported a mean of 3.13 μmol of coenzyme I/g dry dog cardiac muscle (min 2.31 and max 4.15), which corresponds to 689 nmol/g wet wt calculated on the basis of a 78 % water content (15). Such a figure is similar to the NAD value found in the present work for samples taken as noninfarcted tissue.

We are aware that the NADP and $NADPH_2$ figures may involve an important error because of their small magnitude. Also, it is possible that a change in the redox state of these coenzymes may have occurred during the time elapsed since the removal of the heart and the preparation of the tissue extracts. Consequently, the $NADH_2$/NAD values probably do not reflect the in vivo redox state. Nevertheless, since both noninfarcted and infarcted tissues were treated equally, the observed differences between them are valid.

Govier (9) reported a NAD loss of around 75 % at the 2nd h after the arterial occlusion in comparison with zero time level. However, for a similar sample we observed a mean breakdown of around 28 %. This discrepancy seems to reflect the differing conditions used to prepare the extracts. In hearts excised from animals sacrificed some hours before, Michal et al. (14) found that the levels of NAD and $NADPH_2$ diminished and those of $NADH_2$ increased. Again, any differences between our results and theirs are probably a function of important differences between the techniques used to achieve complete anoxia of the whole heart in the dead animal and those used to provoke the electrophysiological damage in a small portion of the left ventricle.

Further work is necessary to study the changes only of the free without including the protein-bound dinucleotides in the infarcted tissue (10).

NAD and $NADH_2$ concentration levels in arterial and coronary sinus blood of normal dogs apparently have not been reported before. Axelrod and Elvehjem (2) published values of coenzyme I only for venous blood which range from 77 to 100 μmol/kg.

The loss of NAD and $NADH_2$ from the infarcted tissue

TABLE 3. *Levels of oxidized and reduced nicotinamide adenine dinucleotides in arterial blood of dogs with myocardial experimental infarct*

	Time, h, After Ligation of the Coronary Artery				
	0	2	4	9	23
	μmol/kg blood with 45% hematocrit				
NAD	35 ± 6	41 ± 7	38 ± 13	41 ± 2	40 ± 1
$NADH_2$	24 ± 6	28 ± 4	30 ± 7	23 ± 3	22 ± 2
NAD + $NADH_2$	59 ± 12	69 ± 11	65 ± 17	65 ± 5	62 ± 3
$NADH_2$/NAD	0.67 ± 0.09	0.70 ± 0.03	0.67 ± 0.04	0.57 ± 0.07	0.57 ± 0.03
	(8)	(5)	(8)	(2)	(2)

Values are means ± SD, with number of animals in parentheses.

TABLE 4. *Levels of oxidized and reduced nicotinamide adenine dinucleotides in coronary sinus blood of dogs with myocardial experimental infarct*

	Time, h, After Ligation of the Coronary Artery				
	0	2	4	9	23
	μmol/kg blood with 45% hematocrit				
NAD	33 ± 3	41 ± 2	36 ± 8	38 ± 3	40 ± 2
$NADH_2$	24 ± 3	28 ± 4	27 ± 8	24 ± 4	23 ± 2
NAD + $NADH_2$	57 ± 9	68 ± 9	61 ± 15	62 ± 7	63 ± 4
$NADH_2$/NAD	0.70 ± 0.11	0.67 ± 0.00	0.69 ± 0.11	0.61 ± 0.04	0.59 ± 0.02
	(8)	(5)	(8)	(2)	(2)

Values are means ± SD, with number of animals in parentheses.

was not reflected in the concentration of these dinucleotides in the whole coronary sinus blood. Alivisatos, Kashket, and Denstedt (1) suggested that the NADase of the rabbit erythrocyte is distributed in the membrane in such a manner that it is capable of reacting with substrate in external medium, and consequently NAD cannot exist in the circulating blood plasma. Bishop, Rankin, and Talbott (4) confirmed that the blood nucleotides do not appear in human plasma. We found that NAD added in vitro (1×10^{-3} M) to a dog blood dilution was rapidly hydrolyzed to nicotinamide (65 nmol/min, ml of blood), whereas the NAD naturally contained in the blood cells was not degraded under such conditions. The decomposition of $NADH_2$ also occurred although at a slower rate (2.6 nmol/min per ml).

The steady loss of the nicotinamide adenine dinucleotides from the cardiac infarcted tissue may be due, then, to a possible leakage toward the bloodstream with subsequent hydrolysis there or to the action of the myocardial NAD glycohydrolase and/or to a defect in their synthesis. Severin, Tseitlin, and Telepneva (16) demonstrated that NAD biosynthesis starts in mitochondria, and it is known that an early damage of these organelles occurs in the infarcted tissue (7).

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NAD glycohydrolase activity in hearts with acute experimental infarction

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NÚÑEZ, ROSARIO, EDMUNDO CALVA, MEYTHA MARSCH, ELISA BRIONES, AND FERNANDO LÓPEZ-SORIANO. *NAD glycohydrolase activity in hearts with acute experimental infarction*. Am. J. Physiol. 231(4): 1173–1177. 1976.—Myocardial ischemia was produced in dogs by occluding the descending coronary artery. NAD decreased in the ischemic tissue taken 2 h after the arterial ligature, and an equivalent amount of nicotinamide was detected instead. A further breakdown occurred when fragments of ischemic and nonischemic tissue were incubated at 37°C. In contrast, NAD concentration remained unchanged for as long as 60 min in incubated fragments from normal heart. When normal tissue was homogenized, an immediate hydrolysis of NAD was observed with the formation of stoichiometric amounts of nicotinamide. An excess of nicotinamide completely inhibited the NAD degradation. The NAD glycohydrolase activity assayed in vitro was similar in normal, ischemic, and nonischemic cardiac homogenates. The conclusions are that the NAD loss in the ischemic heart is due to the tissue NAD glycohydrolase activity and that the cell disorganization provoked by the occlusion of the coronary artery seems to facilitate the reaction between the substrate and the enzyme.

myocardial infarction; ischemic and nonischemic cardiac tissues; dog heart; nicotinamide

STUDIES PREVIOUSLY CARRIED OUT in our laboratory showed that nicotinamide adenine dinucleotides (NAD, $NADH_2$, NADP, and $NADPH_2$) levels decrease in myocardial dog tissue after the ligature of a coronary artery while no significant changes occur in the concentration of these compounds in blood taken from the coronary sinus (16). The purpose of the present work was to seek an explanation for the low levels of NAD in dog ischemic cardiac muscle. We looked for NAD glycohydrolase (NADase) activity analogous to that previously described in hearts from rats (11, 18), guinea pigs (7), mice (20), and rabbits (9).

MATERIALS AND METHODS

Myocardial infarction was provoked in eight mongrel dogs anesthetized with intravenous sodium pentobarbital (30 mg/kg body wt). After tracheal intubation, the animals were artificially ventilated with a Palmer respirator pump. A left thoracotomy was performed at the level of the fourth intercostal space and the left descending coronary artery occluded near its origin. Through the femoral vein the animals received 250 ml of 0.9% (wt/vol) NaCl solution initially, and during the rest of the experiment a 10% (wt/vol) glucose solution at the flow rate of 60–70 ml/h (Holter pump RL75). The dogs were kept anesthetized by sporadic intraperitoneal injections of pentobarbital (128 mg). Sodium penicillin G (1,000,000 U) was administered before surgery and 12 h later. The pericardium and the thorax were sutured only in those animals maintained alive for 24 h. Before excision of the heart the ischemic portion in the anterior wall and the nonischemic region in the posterior wall of the left ventricle were delimited on the basis of unipolar epicardial electrocardiographic records (4). Four of the animals were sacrificed at 2 h and the other four at 24 h after the arterial occlusion.

Hearts from 45 anesthetized dogs were used as normal cardiac tissue.

Normal and infarcted hearts were immersed in ice-cold 0.25 M sucrose immediately after their removal, and then 80 ml of a sucrose solution were perfused to each heart through the left and right coronary arteries. Tissue samples were blotted with filter paper and fragments from them cut with a cork borer.

The stability of endogenous NAD and nicotinamide was studied in whole fragments and in homogenates of myocardium. Fragments of normal, nonischemic and ischemic tissue weighing approximately 1 g were placed in glass cylinders and incubated at 37°C in a water bath. The *time 0* fragments were received in beakers containing 8 ml of an ice-cold mixture of 5% (wt/vol) perchloric acid and 0.125 M sucrose, minced with scissors, and homogenized for 3 min at ice-water temperature. At the completion of the varying incubation times an additional acid homogenate was similarly prepared from each fragment. Homogenates for the study of the stability of both compounds were prepared from normal hearts as follows. A 4-g sample was cut into smaller pieces with scissors in 16 ml of each of the following three ice-cold mediums: 0.25 M sucrose, 0.50 M nicotinamide, and 1.5 M nicotinamide. The concentration of the nicotinamide solutions was adjusted to the tissue sample wet weight (1.5 and 4.5 mmol/g, respectively). The minced tissue was homogenized during a 90-s disintegration period in an all-glass homogenizer immersed in a NaCl ice-cooling mixture. Three milliliters of each suspension were immediately received in an equal volume of ice-cold 10% perchloric acid and additional sam-

ples were inactivated after varying times of incubation. Studies were conducted at various incubation temperatures with endogenous or exogenous NAD.

The activity of NADase was measured in homogenates prepared from normal hearts and from ischemic and nonischemic tissue samples. One gram of tissue was homogenized in 20 ml of ice-cold 0.25 M sucrose and the enzymatic assay was done as follows: 2 ml of 0.10 M sodium phosphate buffer at pH 7.4 and 7 ml of homogenate were preincubated for 3 min at 37°C. The reaction was started by the addition of 1 ml of 0.01 M NAD (Calbiochem, 481911), pH 7. The final pH of the incubation mixture was approximately 7 and the optimal enzymatic activity occurred in the 5.5–8.0 pH range. A 2-ml sample was withdrawn immediately after mixing and discharged into 4 ml of ice-cold 8% perchloric acid. A second sample was inactivated 15 min later.

NAD and nicotinamide were measured in all the acid extracts. NAD was estimated enzymatically (6) by transferring 0.4–1 ml of each acid extract into 10-mm cuvettes containing 2 ml of incubation mixture (0.3 M Trizma base (Sigma, T1503), 0.5 M ethanol (Merck, 200972), pH 10.8). The total volume was 3 ml. A reference with 0.4 ml of 0.33 mM NAD solution was always included. After the initial absorbance at 340 nm was recorded in a Zeiss PMQ 2 spectrophotometer, the reaction was initiated by adding 0.05 ml (50 U) of alcohol dehydrogenase (Sigma, A7011). The steady end-reading absorbance was taken 2–3 min later. Nicotinamide was measured according to the method of Pelletier and Campbell (17), as described by Strohecker and Henning (19). The pH of a 2-ml sample of each acid extract at room temperature was adjusted to the 4.7–5.3 range by adding approximately 2.2 ml of 0.5 M Na_2HPO_4. A 1-ml sample of each of these neutralized extracts was used for the assay, and a standard solution of nicotinamide (Sigma, N3376) containing 10 μg/ml in neutralized perchloric acid was included as a reference. Nicotinamide was stable in ice-cold 5% perchloric acid for at least 5 days, in contrast to the statement of Pelletier and Campbell (17).

RESULTS

Stability of NAD and Nicotinamide in Fragments and Homogenates of Cardiac Tissue

Normal hearts. The levels of NAD and nicotinamide in fragments from normal hearts were well preserved during incubation times of 45 min (four dogs) and as long as 60 min (four dogs) (Fig. 1).

A remarkable decrease of the endogenous NAD and a stoichiometric increase of nicotinamide occurred as soon as the normal cardiac tissue was homogenized (Fig. 1). The NAD degradation rate depended on the temperature of incubation (Fig. 2), and no hydrolysis was observed in the presence of a high concentration of nicotinamide (4.5 mmol/g wet wt tissue) (Fig. 3). Moreover, no changes in NAD and nicotinamide levels were detected when the homogenate was prepared in perchloric acid and then incubated at 37°C.

When exogenous NAD was added to homogenates previously depleted of all the tissue NAD by preincubation at room temperature for 30 min, the rates of degradation at different temperatures were similar to those observed with endogenous NAD (Fig. 2). For these experiments the amount of NAD added to each incubation mixture was of the same order of magnitude as that found in the *time 0* samples of homogenates prepared from normal hearts.

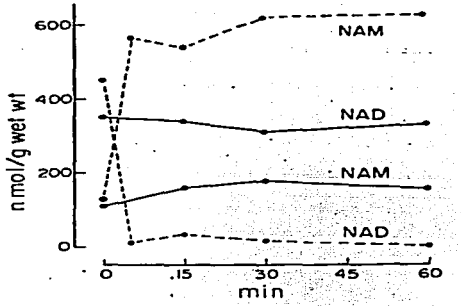

FIG. 1. Stability of endogenous NAD and nicotinamide (NAM) in fragments (——) and in a homogenate (– – – –) incubated at 37°C and prepared from a normal heart. *Dog 7.*

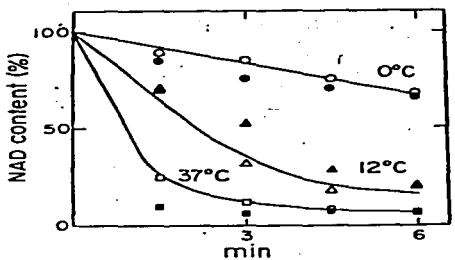

FIG. 2. Rates of degradation of endogenous (open symbols) and exogenous (solid symbols) NAD in homogenates incubated at different temperatures and prepared from a normal heart. *Dog 15.*

Nonischemic tissue. The initial contents of NAD and nicotinamide (nmol/g) in nonischemic tissue from hearts removed 2 and 24 h after occlusion of the artery were similar to those found in normal hearts. However, a certain amount of NAD was hydrolyzed stoichiometrically to nicotinamide when fragments of this nonischemic tissue were incubated at 37°C (Table 1). This finding contrasts with the stability observed in fragments from normal hearts incubated under the same conditions.

Ischemic tissue. The NAD content in ischemic tissue taken from hearts 2 h after ligature was lower than in nonischemic tissue, and the nicotinamide increase was approximately equivalent to the NAD loss. Furthermore, when fragments of this tissue were incubated at

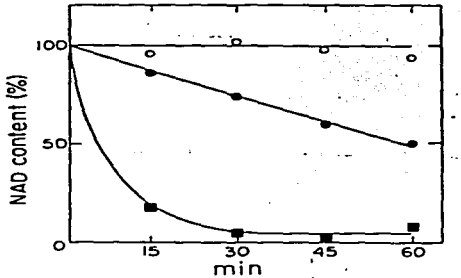

FIG. 3. Inhibition of the endogenous NAD hydrolysis by nicotinamide added to suspension medium before homogenization. Control homogenate (■), homogenates with 1.5 (●) and 4.5 (○) mmol nicotinamide/g tissue. *Dog 17.*

TABLE 1. *Initial content and stability of NAD and nicotinamide in tissue fragments from dog hearts with experimental infarction*

Dog	Tissue Area	Initial Content			Postincubation Content*		
		NAD	Nicotinamide	Total	NAD	Nicotinamide	Total
		2 h after coronary occlusion					
1	Nonischemic	351	105	456	322	172	494
	Ischemic	245	189	434	121	381	502
2	Nonischemic	410	101	511	303	193	496
	Ischemic	341	175	516	123	305	428
		24 h after coronary occlusion					
3	Nonischemic	438	97	535	340	204	544
	Ischemic	41	34	75	25	37	62
4	Nonischemic	401	114	515	306	204	510
	Ischemic	94	86	180	87	100	187

Average values are expressed as nmol/g wet wt from separate analysis of two tissue samples taken from each area. * Incubation period, 30 min at 37°C.

37°C the changes in NAD and nicotinamide levels were more notable than in the nonischemic tissue.

In the ischemic tissue removed 24 h after arterial ligature the initial levels of both compounds were very low, and no further changes were observed after the incubation of fragments of this tissue (Table 1). It is possible that by this time most of the NAD may have been hydrolyzed and the nicotinamide leaked to the bloodstream.

NAD Glycohydrolase Activity

The NADase activity assayed in vitro with an excess of exogenous substrate was of the same order of magnitude in homogenates prepared with normal, 2- and 24-h ischemic and nonischemic cardiac tissue samples. Moreover, the amount of nicotinamide released was always equivalent to the NAD hydrolyzed (Table 2), and no hydrolysis was observed in the presence of a high concentration of nicotinamide (4.5 mmol/g wet wt tissue).

DISCUSSION

Our data demonstrate the presence of NAD glycohydrolase in dog heart homogenates on the findings that no exogenous or endogenous NAD was hydrolyzed when an excess of nicotinamide was added to the incubation mixture, that nicotinamide specifically inhibited the NADase with no action on the NAD pyrophosphatase (15), and the amount of NAD degraded was always equivalent to the nicotinamide formed. Consequently, it seems reasonable to assume that this activity is responsible for the degradation of the endogenous NAD observed in the ischemic cardiac tissue while the infarcted heart remains in the dog and that noted during the incubation of fragments of this tissue at 37°C.

The fact that the specific activity of NADase as determined under optimal assay conditions was similar to homogenates from normal, acutely nonischemic, and ischemic cardiac tissues is remarkable since all of the enzyme activities studied by Gudbjarnason et al. (10) showed significant changes in ischemic tissue extracts 24 h after coronary artery occlusion.

The extreme conditions of anoxia and ischemia under which fragments of normal hearts were maintained in vitro at 37°C were not harmful enough to stimulate degradation of the endogenous NAD during a short period of incubation, whereas the partial intracellular disorganization occurring in the ischemic tissue after the arterial ligature (5, 13) and the total mechanically induced disruption occurring during the preparation of the homogenized suspension seem to facilitate the reaction between the endogenous substrate and the enzyme.

What might be the possible mechanisms to account for the stability of endogenous NAD in normal tissue and its hydrolysis in ischemic myocardium given that NADase activity appears to be of the same order of magnitude in both?

According to Bernofsky and Pankow (1) the NADase tested in vitro induces a rapid breakdown of free NAD and has only a minimal effect on the enzyme-bound

TABLE 2. *NAD glycohydrolase in homogenates prepared from normal and infarcted dog hearts*

Dog	Tissue Area	Enzyme Activity, μmol/g wet wt, 15 min	
		NAD consumed	Nicotinamide produced
	2 h after coronary occlusion		
1	Nonischemic	15	17
	Ischemic	11	15
2	Nonischemic	9.4	11
	Ischemic	9.5	12
	24 h after coronary occlusion		
3	Nonischemic	12	10
	Ischemic	12	12
4	Nonischemic	11	9.8
	Ischemic	12	11
	Normal hearts (8 animals)	7.3–14	7.6–15

dinucleotide. Therefore, the binding of the NAD to proteins might be an explanation for its stability in normal tissue, as has been proposed by Kaplan (14). However, two previous observations seem to invalidate this possibility. Green et al. (8) reported that essentially all of the NAD bound to crystalline rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was rapidly hydrolyzed by a highly purified mammalian NADase, and Bernofsky and Pankow (1) found that 80% of the intracellular NAD occurs unbound in the rabbit skeletal muscle. In the present work we have shown that the endogenous NAD degraded by the NADase during the homogenization of the tissue seems to exist in the unbound protein form, since the time periods of hydrolysis at different temperatures were similar to those observed with exogenous free NAD. Consequently, we may conclude that the possible unbinding that may occur during the infarction process or during the mechanical disruption of the tissue is not the primary cause of the rapid degradation of the endogenous NAD observed under such conditions.

Bock et al. (2) have presented evidence that the microsomal NADase of the Ehrlich ascites cells is mainly located at the plasma membrane and have suggested that this location could protect the intracellular NAD from being degraded by the enzyme. Preliminary studies by our group indicate that the NADase activity is not present in the cytosol but rather that it is principally to be found in two microsomal subfractions isolated from dog heart homogenates. On the other hand, Jacobson and Kaplan (12) detected that 90% of the rat liver cell NAD is in the soluble fraction and only 3% in the microsomes. Consequently, if we assume a similar distribution of the NAD in the normal dog cardiac cell, a topological separation would seem to exist between the major portion of the oxidized form of this coenzyme and the NADase. Therefore, we suggest that it is possible that the reaction between the endogenous NAD and the enzyme is facilitated in vivo by local tissue alterations occurring after occlusion of the coronary artery and in vitro by the mechanical disruption of the myocardium. Further work is necessary to elucidate how this facilitation occurs in the ischemic cardiac tissue.

On the basis of the present results we can explain our previous findings (16) by assuming that the low levels of nicotinamide coenzymes in ischemic cardiac tissue are due mainly to a hydrolytic degradation catalyzed by the NADase and facilitated by the tissue disorganization. The depletion of the NAD pool may decrease the activity of those dehydrogenases depending on NAD as coenzyme and those requiring it for stability and protection against proteolytic inactivation (8).

Braasch et al. (3) showed that in nonischemic myocardium the pattern of systolic contraction persists and may even show an increase in amplitude, and they suggested that the increased activity of some of the enzymes could be in response to increased energy requirements. On the other hand, Jennings et al. (13) found that the fine structure of myocardium from the nonischemic region appeared unchanged and conformed in general to the appearance of normal cardiac tissue. It is difficult with the present data to see the significance of the higher rate of NAD hydrolysis that we observed during the incubation of nonischemic tissue fragments and to relate this finding to the suggestion that these portions of the heart may be able to meet additional energy requirements by way of increased aerobic energy metabolism (3).

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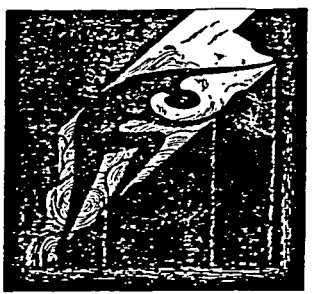

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NAD GLYCOHYDROLASE ACTIVITY IN DOG CARDIAC TISSUE

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Abstract—1. Dog heart tissue suspension hydrolyzes NAD, NADP and NMN, and releases nicotinamide stoichiometrically.

2. Maximum activity was observed at 50°C and the activation energy was 10 kcal/mol.

3. Optimum pH range was 6.2–7.6.

4. Compounds with adenine–ribose moiety increased the enzymatic activity.

5. Nicotinamide released during incubation produced reaction nonlinearity.

6. K_m for NAD and NADP were about the same; V_{max} was higher for NAD. Similar findings have been reported for rabbit heart.

7. Dog enzyme appears to be more sensitive than the rabbit enzyme to noncompetitive inhibitors.

8. Pyrophosphatase activity was not detected in dog heart in contrast to rabbit and rat heart preparations.

INTRODUCTION

In an earlier study (Núñez *et al.*, 1976) it was shown that NAD* glycohydrolase activity produces a decrease in the oxidized form of the nicotinamide-adenine dinucleotide and an increase in free nicotinamide in dog ischemic cardiac tissue following the occlusion of the descendent branch of the left coronary artery.

The purpose of the present work has been to study this activity in the homogenized suspension of normal myocardial tissue from dogs (*Canis familiaris*).

MATERIALS AND METHODS

Mongrel dogs weighing 15–20 kg were sacrificed by electric shock. The heart was received in ice-cold 0.25 M sucrose (Baker 4072) as soon as it was excised, and was perfused with the solution through both coronary arteries. Ventricular fragments blotted with filter paper and weighing around 1 g were stored in a deep-freezer (−70°C). NADase activity was well preserved for at least 4 months.

Homogenized suspensions with 20–60 mg of wet weight tissue per ml of 0.25 M sucrose were prepared in a Potter–Elvehjem homogenizer immersed in ice. Three 30-sec periods of mechanical stirring were used to disrupt the tissue.

The incubation mixture to assay the NADase activity was prepared at room temperature with 1.0 ml of 0.10 M Na-phosphate (Merck 206586 and Merck 206346) buffer at pH 7.4, 1.0–3.5 ml (2.5–10 mg of proteins) of tissue suspension filtered through two layers of gauze, and the volume of 0.25 M sucrose solution necessary to complete 4.5 ml. The mixture was left 5 min at room temperature and 3 min at 37°C, then the reaction was started with the addition of 0.5 ml of 10 mM NAD (Calbiochem 481911) solution at pH approx 7.4. As soon as the mixture was well mixed, a 2-ml sample was transferred to a test tube containing 4 ml of ice-cold 8% perchloric acid (Baker 9652) solution, and the tube with the remaining mixture was placed in a 37 ± 0.1°C water bath. A second sample was taken 15 min later. Both the NAD and the nicotinamide concentrations were always determined in duplicate in the acid supernatant solutions stored at 4°C. To measure NAD, 50 units of alcohol dehydrogenase were added to a spectrophotometer cell containing 0.4 ml of the sample, 0.6 ml of 5% perchloric acid, and 2.0 ml of incubation mixture with 0.6 M trizma base (Sigma T 1503) and 1.0 M ethyl alcohol (Merck 200972) at pH 10.8. Nicotinamide was assayed colorimetrically with cyanogen bromide (Núñez *et al.*, 1976). When either NADP (Calbiochem 481971) or NMN (P-L Biochemicals 6000) was the substrate the glycohydrolytic activity was determined only by the amount of nicotinamide liberated. None of the three substrates was hydrolyzed in the perchloric acid medium used to inactivate the enzyme. Proteins were measured with the Folin–Ciocalteu reagent (Merck 9001) (Oyama & Eagle, 1956) in the tissue diluted samples previously treated with 5% Na-deoxycholate (Merck 6504); bovine serum albumin (Sigma A 4378) was used as the reference. All exceptions to the conditions described above are so indicated.

The specific activity is expressed in nmol of NAD consumed or nicotinamide liberated during 15 min of incubation per mg of protein in an assay medium containing 0.02 M of sodium phosphate buffer and substrate at a concentration high enough to saturate the enzyme (1 mM), and at the pH and temperature indicated in each experiment. The velocity v is the specific activity referred to 1 min of incubation, and measured at 37°C and at pH 7.3, with substrate concentrations lower than the enzyme saturation level.

RESULTS

Effect of temperature on the activity and stability of the enzyme

The optimum temperature to hydrolyze the coenzyme NAD incubated with the dog cardiac tissue suspension was 50°C (Fig. 1). In the present study, 37°C was always used as the temperature of incubation. The free energy of activation cal-

* Abbreviations: Nam, nicotinamide; NMN, nicotinamide mononucleotide; NAD, nicotinamide-adenine dinucleotide; NADP, nicotinamide-adenine dinucleotide phosphate; NADase, NAD glycohydrolase; EDTA, ethylenediamine tetra-acetic acid disodium salt; AMS, adenosine-5'-monosulfate; AMP, ADP, ATP, adenosine-5'-mono-di-,triphosphate.

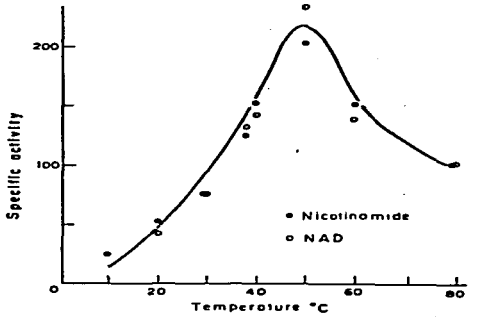

Fig. 1. Effect of temperature on NADase activity. The enzyme specific activity is expressed as nmole of NAD hydrolyzed or nicotinamide liberated per 15 min per mg of protein.

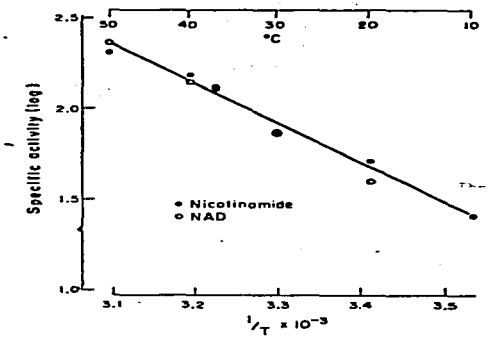

Fig. 2. Arrhenius plot to calculate the free energy of activation.

culated from the Arrhenius plot between 10 and 50°C (Fig. 2) was approx 10 kcal (42 kJ) and the Q_{10} value 1.8. The NADase activity assayed at 37 and 50°C, respectively, was similar before and after the tissue suspension was heated for 15 min at 50°C (Table 1).

Effect of pH

Two series of buffer solutions were used: one prepared with Na-phosphates and the other with maleate–Tris (Sigma M 0375; Sigma T 1503). The specific activity values were similar in both series and the highest figures were observed between 6.2 and 7.6 (Fig. 3). The NADase activity assayed in two phosphate buffers, one 0.01 M and the other 0.20 M, was similar in both cases to that found in the 0.02 M solution, which was the concentration normally used.

Table 1. Effect of heating the dog cardiac tissue suspension at 50°C for 15 min before incubation

Condition of the sample	Incubation temperature (°C)	Specific Activity (nmol/15 min/mg protein) NAD consumed	Nam liberated	Ratio NAD/Nam
Control	37	118	113	1.0
	50	203	189	1.1
Treated	37	118	107	1.1
	50	206	189	1.1

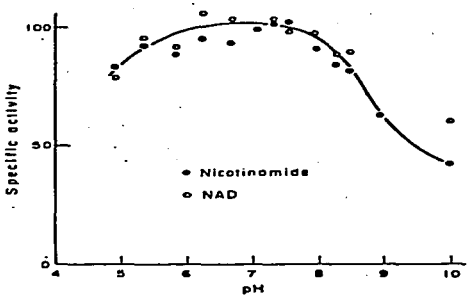

Fig. 3. Influence of pH on NADase activity. The pH values were measured at room temperature in the incubation mixture. Nonenzymatic breakdown occurs above pH 10.

Effect of incubation time and tissue concentration

The velocity of the NAD hydrolysis at 37°C and pH 7.3 in phosphate buffer was constant during the first 3 min with 0.5–4.6 mg of proteins per ml of incubation mixture. This initial velocity was maintained for as long as 15 min if the tissue concentration did not exceed 1.8 mg per ml (Fig. 4). Consequently, where it was necessary to add larger quantities of proteins the time of incubation was shortened. When the NAD concentration was 4 mM instead of 1 mM, the progress of the reaction was modified in neither low (0.9 mg/ml) nor relatively high (4.6 mg/ml) protein concentrations (Fig. 5). On the other hand, the velocity did not show any increment when a new amount of NAD was added 1 hr after the start of the reaction (Fig. 6), but the initial velocity was recovered when NAD was added to a new suspension prepared from a tissue pellet separated by centrifugation from a mixture previously incubated for 1 hr (Fig. 6).

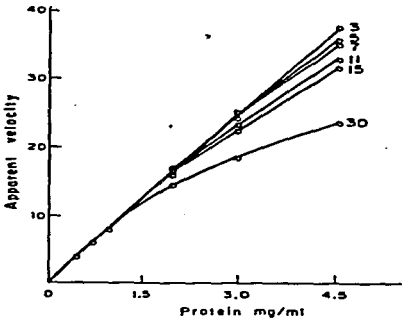

Fig. 4. Enzymatic activity as a function of different concentrations of cardiac tissue in the assay medium. The numbers at the end of the plots represent the incubation time in min. The apparent velocity is expressed as nmol of nicotinamide liberated per ml of incubation mixture.

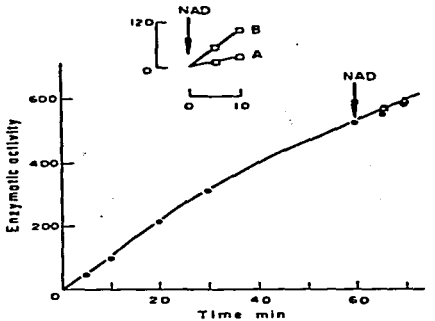

Fig. 6. Effect on the reaction rate of NAD added to the incubation mixture. Main graph: the time course with (Δ) and without (●) the addition of more substrate. Inset: the time course in the original assay medium (plot A) and in a new resuspension of the tissue (plot B) after 1 hr of incubation.

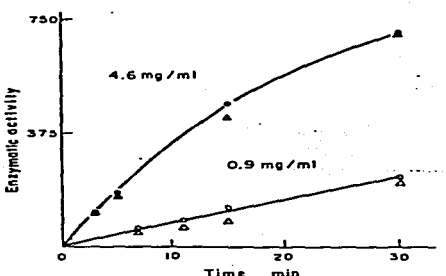

Fig. 5. The time course of nicotinamide liberated by NAD hydrolysis in the presence of either a low (open symbols) or a high tissue concentration (solid symbols). Substrate was used at 1 mM (circles) and 4 mM (triangles). The enzymatic activity is expressed as nmole of nicotinamide liberated per ml of incubation mixture.

K_m and V_{max} values for the substrates NAD, NADP and NMN

To determine these values for NAD and NMN we used 1.1 mg of proteins per ml of incubation mixture and 0.10, 0.15, 0.20, 0.35 and 0.50 mM of substrate. The incubation times were 7.5, 8, 10, 10 and 10 min, respectively. For NADP, the proteins were 3.5 mg/ml and the concentrations of substrate 0.10, 0.20, 0.30, 0.40, 0.50 and 1.00 mM, with incubation times of 3 min for the first substrate concentration and 5 min for the rest. The kinetic constants were calculated from the graphs: $1/v$ vs $1/S$ (Lineweaver–Burk), S/v vs S (Hanes) and v vs v/S (Eadie–Hofstee) (Fig. 7). The K_m values for NAD were 6.7–7.1; for NADP, 4.6–5.6; and for NMN, 12–13.5 × 10^{-5} M. The maximum velocity of hydrolysis was 9.2–9.5 for NAD, 7.1–7.6 for NADP, and 6.7–7.0 for NMN, nmoles per min per mg of proteins (Table 2).

Inhibition studies

The K_i value for nicotinamide was determined under the experimental conditions used to measure the K_m for NAD and was calculated by five graphic methods (Figs. 7 and 8). It was independent of the three concentrations (0.3, 0.6 and 1.5 mM) of nicotinamide (Calbiochem 4813) used (Table 3).

The effect of the isonicotinic acid hydrazide (Sigma I 3377) was studied with 1.1 mg of protein per ml; 0.10, 0.15, 0.20, 0.35, 0.50, 0.70 and 1.0 mM of NAD; and three different concentrations of this inhibitor (0.10, 0.30 and 0.50 mM). The incubation times were similar to those used in the case of nicotinamide. The values of K_i was 0.13–0.17 × 10^{-3} M with the lowest concentration and 0.24–0.26 × 10^{-3} M with the other two concentrations of hydrazide (Fig. 9, Table 4).

When theophylline (Calbiochem 5861) was added at the final concentration of 0.37 mM to an incubation mixture containing 1.4 mg of proteins per ml and NAD 1 mM, it inhibited 48% of the NADase activity (Fig. 10).

NAD/nicotinamide ratio. NAD pyrophosphatase

The ratio NAD consumed/nicotinamide liberated was approx 1 (0.9–1.1) in all the experiments except when the pH of the incubation mixture was 10 (ratio equal to 1.45).

NAD pyrophosphatase activity (EC 3.6.1.9) was studied in homogenized tissue suspensions prepared in one case with dog heart and in the other with rat liver. Both preparations were incubated

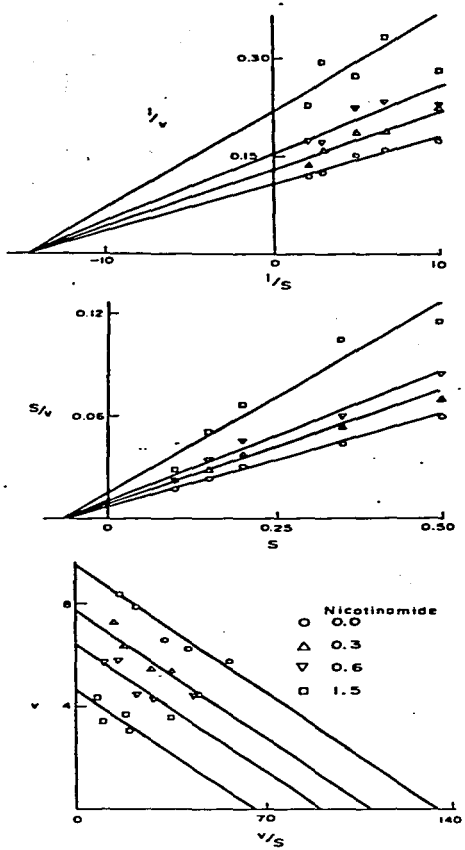

Fig. 7. Lineweaver-Burk, Hanes and Eadie-Hofstee plots for the enzymatic hydrolysis of NAD in the presence of nicotinamide at three different concentrations. Velocity is expressed as nmole of NAD consumed per mg of protein per min. Substrate concentration is $M \times 10^{-3}$.

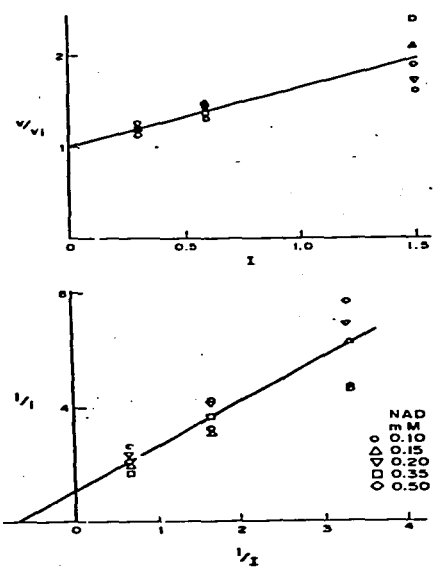

Fig. 8. Dawes and Webb plots. Same data as in Fig. 7.

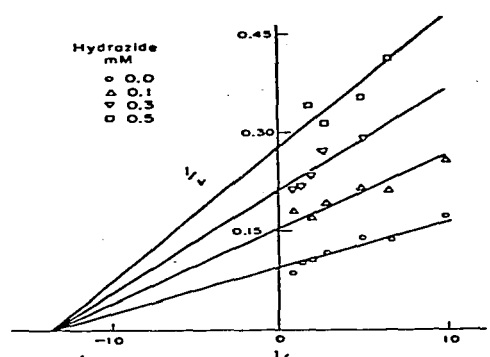

Fig. 9. Lineweaver-Burk plot for the enzymatic hydrolysis of NAD in the presence of isonicotinic acid hydrazide at three different concentrations.

with $MgCl_2$ (Merck 5833) 5 mM and nicotinamide 4.5 mmol per g of tissue (0.43 and 0.27 mM for the heart and liver suspensions, respectively) to stimulate the pyrophosphatase activity and to inhibit the NAD glycohydrolase; and with EDTA (Sigma ED 2 SS) 1 mM and nicotinamide to inhibit both

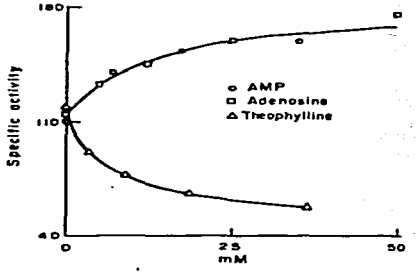

Fig. 10. Effect of AMP, adenosine and theophylline, on the enzymatic hydrolysis of NAD.

Table 2. Kinetic parameters of NADase activity from dog cardiac tissue with three nicotinamide nucleotides

Graphic method	Km ($M \times 10^{-5}$) NAD	NADP	NMN	Vmax (nmol/min/mg protein) NAD	NADP	NMN
1/v: 1/S	6.7	5.6	13.5	9.3	7.6	6.9
S/v: S	6.8	5.0	12.0	9.1	7.1	6.7
v: v/S	7.1	4.6	12.8	9.5	7.1	6.9

Table 3. K_i values of NADase activity from dog cardiac tissue with nicotinamide at different concentrations

Graphic method	K_i ($M \times 10^{-3}$) A*	B*	C*
1/v : 1/S	1.4	1.4	1.4
S/v : S	1.4	1.6	1.5
v : v/S	1.3	1.2	1.4

* A, B and C mean 0.3, 0.6 and 1.5 mM of nicotinamide. K_i values from v/v_i: I (Dawes) and $1/i$: $1/I$ (Webb) graphs were 1.5 and 1.4, respectively.

Table 4. K_i values of NADase activity from dog cardiac tissue with isonicotinic acid hydrazide at different concentrations

Graphic method	K_i ($M \times 10^{-3}$) A*	B*	C*
1/v : 1/S	0.16	0.24	0.26
S/v : S	0.13	0.25	0.26
v : v/S	0.17	0.25	0.26

* A, B and C mean 0.1, 0.3, 0.5 mM of hydrazide.

Table 5. Effect of some adenylic compounds on the NADase activity from dog cardiac tissue

Addition 10 mM	Specific activity (nmol/15 min/mg protein)
None	103
Adenine	107
Adenosine	137
AMS	153
AMP	126
ADP	148
ATP	135
ADP-ribose	110

Table 6. NADase and NAD pyrophosphatase activities in dog and rat tissue preparations

Additions	NAD consumed (nmol/15 min/ml of incubation mixture) Dog heart	Rat liver
None*	805	1655
$MgCl_2$ + Nam*	8	221
EDTA + Nam*	21	66

Time of incubation at 37°C: * 5 min; † 15 min.

enzymes (Emmelot *et al.*, 1964). The incubations were done with 10 mg of proteins of each tissue for 15 min to seek for the pyrophosphatase activity. In the dog heart preparations NAD was hydrolyzed exclusively by the glycohydrolase activity, and in the case of the rat liver suspension pyrophosphatase was responsible for only 9% of the total NAD hydrolyzed (Table 6).

Effect of adenylic compounds

None of these compounds at the concentration of 1 mM showed an effect on the NADase activity. At a higher concentration (10 mM) all the compounds with the adenine–ribose moiety activated the enzyme except the ADP–ribose (Table 5). Adenosine and AMP had an equivalent effect, and the highest activation due to either one was observed at 50 mM concentration (Fig. 10). The following compounds were used: adenine (Sigma A 8626), adenosine (P-L Biochemicals 1400), AMS (Calbiochem 1183), AMP (Sigma A. 2127), ADP (Sigma A 0127), ATP (Sigma A 3127) and ADP–ribose (Calbiochem 117615).

DISCUSSION

Hydrolysis of the coenzyme NAD, observed during its incubation with a homogenized suspension of dog heart muscle, appears to be due to the NAD glycohydrolase activity present in this tissue. While Lowry *et al.* (1961) showed that NAD in Tris-buffer solution at pH 7.3 was only sightly hydrolyzed (approx 1%) during 15 min at 60°C, we found a 63% degradation at 50°C, which was the temperature of maximum enzymatic activity. Moreover, we proved both the stability of the

dinucleotide in the incubation mixture and the nonsignificant change of the pH value in the range of temperatures used.

A discontinuity in the slope of the straight line in the Arrhenius plot, which would indicate a transition temperature for NAD enzymatic hydrolysis, was not observed. The value of the free energy of activation, determined at pH 7.3 in phosphate buffer, was similar to that found in a purified preparation of pig liver (Dickerman *et al.*, 1962).

The NADase activity was preserved when the tissue suspension was heated for 15 min at 50°C. This suggests that had there been conformational changes in the enzyme molecule they were reversed under the assay conditions. In contrast with our results, Prikhod'ko *et al.* (1974) reported a 30% inactivation when the microsomal fraction isolated from rabbit heart muscle was heated at 53°C for 15 min.

With the dog cardiac tissue suspension, the highest NADase specific activity was observed at approximately pH 7 in both the phosphate and the Tris–maleate buffer, whereas in the case of the rabbit heart homogenized suspension (Severin *et al.*, 1963) and the microsomal fraction (Prikhod'ko *et al.*, 1974), the optimum pH was 6.2. In this last tissue, in addition to the NAD glycohydrolase activity, which was the main pathway for the degradation of this dinucleotide, a nonglycohydrolytic activity (probably an NAD pyrophosphatase) was found with an optimum pH of 7.5 (Severin *et al.*, 1963). In contrast, since in all the assays of the 12 incubation mixtures used between the pH range 5–9, the ratio NAD/nicotinamide was approx 1, it may be concluded that the only enzymatic activity present in the dog preparation was the NAD glycohydrolase. On the other hand, the dinucleotide degradation did not depend on either the presence or the concentration of the phosphate.

The initial velocity of the NAD hydrolysis was not modified when the substrate added was at the concentration of 4 mM instead of 1 mM. Consequently, this last concentration (equivalent to 14 times the K_m value) was high enough to saturate the enzyme contained in 4.6 mg of protein of homogenized tissue suspension per ml of incubation mixture.

Since the observed per cent inhibition corresponds quantitatively to the nicotinamide concentrations reached successively in the incubation mixture, it may be concluded that the nicotinamide released from NAD produced the fall in reaction velocity during the incubation. Furthermore, the initial reaction rate was recovered when the tissue was resuspended in a fresh incubation medium. This finding also shows that the enzyme inhibition caused by the nicotinamide is freely reversible since the inhibition disappeared as soon as the supernatant solution containing the nicotinamide was separated from the tissue. Since the inital rate was not regained when more NAD was added 1 hr after the reaction was started, the slowness of the reaction may not be attributed to a lack of substrate.

The K_m value in the dog cardiac preparation was slightly lower for NADP than for NAD, while a much higher value resulted for NMN. On the other hand, Prikhod'ko *et al.* (1974) using the heart rabbit microsomal fraction found a K_m value for NADP significantly lower than that for NAD; the ratio K_m(NAD)/K_m(NADP) was 5.4 in contrast to 1.4 for the dog heart homogenized suspension. The maximum velocity value for NAD was higher than that for NADP both in dog (1.3 times) and rabbit (1.8 times) preparations. According to Prikhod'ko *et al.* (1974), the additional phosphate group present in the NADP molecule may increase the electrostatic attraction between this substrate and a cationic region in the active center of the enzyme.

In the dog cardiac preparation, the inhibition of the NAD hydrolysis by nicotinamide was reversible and noncompetitive, and that produced by the isonicotinic acid hydrazide was also noncompetitive. However, the affinity of the enzyme for the hydrazide was six times greater than that for nicotinamide. Also noncompetitive was the inhibition produced by these two compounds in the rabbit heart microsomal fraction, but the affinity for nicotinamide was twice that for the hydrazide (Prikhod'ko *et al.*, 1974). Furthermore, the NADase enzyme present in the dog heart seems to be more sensitive than that found in the rabbit heart—40 times in the case of the hydrazide and three times in the one of nicotinamide. Zatman *et al.* (1953) have proposed that the enzymatic inhibition depends on the competition between the water and nicotinamide for the enzyme–ADP ribose complex. If the amide is present in excess, the velocity of the reaction between the free nicotinamide and the enzymatic complex is equal to the NAD degradation velocity observed in the absence of the inhibitor, and, consequently, an apparent inhibition of the enzyme results. The mechanism of inhibition caused by the isonicotinic acid hydrazide seems to be similar. However, the hydrazide in reacting with the enzymatic complex will form a NAD analogue so tightly bound to the enzyme that no more interaction between the enzyme and NAD will occur (Prikhod'ko *et al.*, 1974). Boriskina & Tseitlin (1972) showed the synthesis of the 3-acetylpyridine-adenine dinucleotide, an NAD analogue, in a rabbit heart preparation with a high NADase activity. This finding also supports the idea that the nicotinamide and the hydrazide inhibit the enzyme by reacting with the enzyme–ADP ribose complex.

We cannot explain the apparent greater affinity ($K_i = 0.15 \times 10^{-5}$ M) of the enzyme for the isonicotinic acid hydrazide when this inhibitor is at a low concentration (0.1 mM) in the incubation mixture. Probably the K_i value (0.25×10^{-3} M) found at the two higher concentrations (0.3 and 0.5 mM) is nonsignificantly different from 0.15×10^{-3} M, or these concentrations may actually modify the conformation of the enzyme and make it less sensitive to the hydrazide.

The adenine–ribose group by itself (adenosine) or as a part of other molecules (AMS, AMP, ADP and ATP) seems to be responsible for the NADase activation. Moreover, the presence of additional phosphate or sulfate moieties in the

molecule of the activator compound appears to be irrelevant. ADP-ribose, one of the products from the NAD hydrolysis which has the adenine–ribose group in its molecule, did not modify the enzymatic activity, wheras nicotinamide, the other product of the reaction, caused an 87% inhibition. In contrast to nicotinamide, ADP-ribose does not appear to react with the enzyme–ADP ribose complex (Zatman *et al.*, 1953).

Haugaard *et al.* (1960) showed a high degree of NAD pyrophosphatase activity in a rat heart suspension prepared in a medium containing KCl, $MgCl_2$ and phosphates which was incubated with KCl, $MgCl_2$, glucose and nicotinamide. Also, Severin *et al.* (1963) reported that 32% of NAD hydrolysis was due to non-nucleosidase activity in a rabbit heart suspension prepared in KCl and incubated in a phosphate buffer at pH 7.3. On the other hand, Hulsmans (1961) proved an absence of NAD pyrophosphorylase activity in all fractions isolated from a rat heart homogenized suspension. We, however, did not find any NAD pyrophosphatase activity in the dog heart preparation incubated under the conditions described by Emmelot *et al.* (1964). This finding and the fact that the NAD/nicotinamide ratio was always approx 1, lead us to conclude that under the present experimental conditions the only enzymatic activity responsible for the NAD hydrolysis was the NAD glycohydrolase.

The enzymatic activity detected in dog cardiac tissue suspensions differs apparently from that found in rabbit heart preparations in the following aspects: the NAD is hydrolyzed exclusively at the glycosidic nicotinamide–ribose linkage; the optimum degradation occurs in a wider pH zone; the K_m value is not significantly different for NAD and NADP; and the affinity for the specific enzyme inhibitors is very much higher. Since the three nicotinamide nucleotides are hydrolyzed by the dog myocardial preparation, further investigation is needed to determine if there is a common enzyme or different ones specific for each substrate and where they are located in the cell. Finally, in view of the lack of information about the role of this enzyme in the normal metabolism of the human heart and during clinically acute myocardial ischemia and in view of the apparent differences detected among the species already investigated it seems appropriate to study this enzyme in hearts of humans beings or animals phylogenetically closer to us.

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NICOTINAMIDE ADENINE DINUCLEOTIDE DEGRADATION IN INFARCTED CARDIAC MUSCLE

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SUMMARY

Nicotinamide adenine dinucleotides (NAD, $NADH_2$, NADP, and $NADPH_2$) levels decrease in myocardial dog tissue after the ligature of a coronary artery branch. The activity of a glycohydrolytic enzyme acting on NAD and releasing nicotinamide in an equivalent amount was of the same order of magnitude in infarcted tissue, irrespective of the time elapsed after the coronary artery occlusion, as it was in normal tissue. Most of the NAD contained in normal heart muscle was hydrolyzed as soon as the tissue was disrupted in a homogenizer, whereas no hydroylsis occurred when the whole fragment was incubated for 1 hour. The enzymatic activity was found mainly in a membranous fraction separated at 17,000 × *g* by differential centrifugation. Acid phosphatase, K^+-activated phosphatase, and NA^+-K^+-ATPase specific activities were greater in this fraction. It is suggested that the structural disorganization of the heart elicited either *in vitro* or during the infarction process determines the conditions for a reaction between the enzyme which is localized in the membranes and the NAD which is mainly in the cytosol.

INTRODUCTION

In previous work (Calva *et al.*, 1966; Nuñez *et al.*, 197), we showed that mitochondria isolated from infarcted heart muscle and incubated with β-hydroxybutyrate exhibit a low oxygen consumption rate, whereas succinate is oxidized at a normal rate. Furthermore, the P/O values measured with either of these two substrates decrease progressively, and the nicotinamide adenine dinucleotides[1] content diminishes in both the whole tissue and the isolated mitochondria. We suggested that the function of the respiratory chain linked to the oxidation of β-hydroxybutyrate may have been impaired at the coenzyme level as a consequence of the loss of the nicotinamide adenine dinucleotides. We

[1] Abbreviations used are: NAD, nicotinamide adenine dinucleotide; $NADH_2$, reduced NAD; NADP, nicotinamide adenine dinucleotide phosphate; $NADPH_2$, reduced NADP; NMN, nicotinamide mononucleotide.

proposed three possible explanations for the loss: a) a leakage of the tissue nicotinamide coenzymes toward the bloodstream; b) an enzymatic breakdown of these compounds in the myocardial tissue itself; and c) a defect in their synthesis. With regard to the first of these possible explanations, no significant changes were observed in the level and the ratio of NAD and $NADH_2$ in the coronary sinus blood taken after ligation of the coronary artery branch (Nuñez *et al.*, 1974). However, an enzyme which hydrolyzes NAD added to blood was detected in the dog white cells (unpublished results). Hence, we concluded it was possible that the myocardial nicotinamide adenine dinucleotides are released from the infarcted tissue and then hydrolyzed in the blood. To investigate the second possible explanation, an enzymatic activity in dog heart homogenates was measured. It was found that NAD, $NADH_2$, NADP, $NADPH_2$, and NMN were hydrolyzed between the pryidine and ribose moieties (unpublished results). It was also observed that most of the NAD contained in normal heart muscle was decomposed as soon as a tissue fragment was disrupted in a homogenizer, whereas no hydrolysis occurred when the whole fragment was incubated at 37°C for 1 hour.

The present report deals with the NAD glycohydrolase activity in infarcted myocardial tissue and with the subcellular distribution of this enzyme in normal cardiac tissue.

METHODS

Normal hearts were obtained from eight mongrel dogs anesthetized with intravenous pentobarbital or sacrificed by electrocution. Infarcted hearts were prepared in four anesthetized dogs by ligature of the left descending coronary artery after the pericardial sac was opened. Before excising the heart, the extent of infarcted tissue was measured by the electrocardiographic changes appearing in the unipolar epicardial records (Calva *et al.*, 1965). Animals were kept anesthetized by intraperitoneal injections of pentobarbital at intervals and received 250 ml of saline solution followed by a 10% glucose solution (60–70 ml/hour) through the femoral vein. Subsequently, two of the four animals were sacrificed 2 hours after occlusion, and the other two at 24 hours after occlusion.

Both normal and infarcted hearts were immersed immediately in ice-cold 0.25 M sucrose solution and perfused through both left and right coronary arteries with the sucrose solution.

Tissue homogenates

Left ventricle fragments were blotted with filter paper, and a tissue suspension was prepared with a glass Potter-Elvehjem homogenizer at a ratio of 0.5 g to 20 ml of ice-cold 0.25 M sucrose solution.

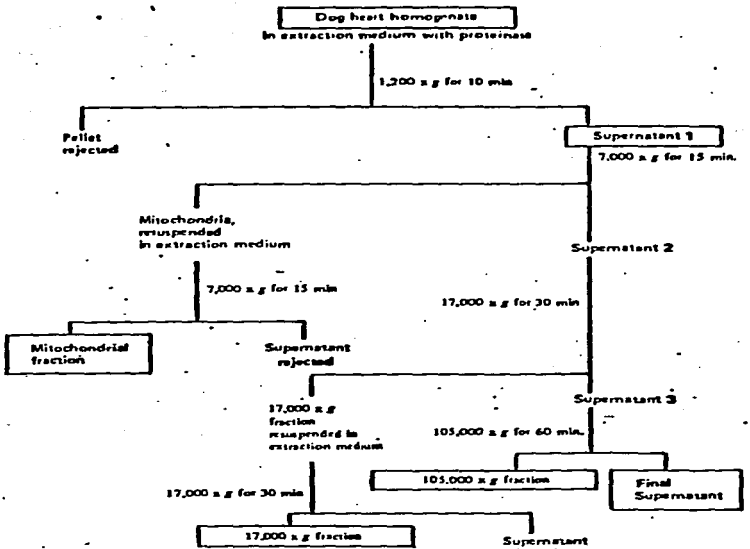

Figure 1. Flow sheet for preparation of subcellular fractions from dog heart homogenate. NAD glycohydrolase activity was measured in those fractions enclosed in a square. Relative centrifugal forces are average × *g* values.

Tissue fractionation

The homogenate was prepared, as described elsewhere (Calva *et al.*, 1965), in an extraction medium containing around 2,250 units of *B. subtilis* proteinase for each 2.5 g of myocardial left ventricle. Subcellular fractions were obtained by differential centrifugation (Fig. 1).

Measurement of NAD glycohydrolase activity

This activity was estimated as follows: 0.50 ml of 0.10 M sodium phosphate buffer, pH 7.4, and 1.0–1.75 ml of tissue fraction suspended in extraction

medium were preincubated for 3 min at 37°C; 0.25 ml of 0.01 M NAD solution was then added. A sample of 1 ml of this mixture was withdrawn immediately after mixing and emptied into 2 ml of ice-cold 8% perchloric acid solution. After 15 min of incubation, a second sample was similarly treated. The NAD was measured directly in the acid extract with lithium lactate and lactate dehydrogenase, the nicotinamide was measured according to the technique of Strohecker and Henning (1966) after the pH of the tissue extract was adjusted to 4.6–5.7 by adding 0.5 M disodium hydrogen phosphate.

Normal hearts

Cylindrical pieces of the left ventricle weighing around 1g were incubated at 37°C. One piece was homogenized immediately in ice-cold 10% perchloric acid solution and the others at the end of 15, 30, and 60 min of incubation.

Homogenates were prepared in ice-cold sucrose solution (1 g in 3 ml) and incubated at 37°C. Proteins were precipitated by the addition of cold percholoric acid at 0, 5, 15, 30, and 60 min of incubation.

Infarcted hearts

Cylindrical fragments of the left ventricle were taken from the anterior wall (infarcted tissue) and from the posterior wall (control tissue), selected on the basis of the electrocardiographic records. The same procedures used on cylindrical pieces of normal heart were also performed on infarcted hearts, but only at time zero and after 30 min of incubation.

Assay of other enzymatic activities

The following were used as marker enzymes: cytochrome *c* oxidase (Wharton and Tzagoloff, 1967), Na^+–K^+–Mg^{++}-ATPase and Mg^{++}-ATPase (Kidwai *et al.*, 1971b), K^+-activated *p*-nitrophenylphosphatase (Kidwai, Radcliffe, and Daniel, 1971a), and acid *p*-nitrophenylphosphatase (Ostrowski and Tsugita, 1961).

RESULTS

Stability of NAD and nicotinamide in the cardiac tissue

The tissue contents of NAD and nicotinamide were well preserved in the cylindrical fragments of normal heart. In contrast, a remarkable decrease of NAD and a stoichiometric increase in nicotinamide occurred during incubation of the homogenate (Table 1).

In the infarcted cardiac tissue excised 2 hours after the coronary occlusion, the initial content of NAD was lower than that found in the control tissue whereas the level of nicotinamide appeared higher. The sum of the levels of the two compounds was similar in both tissues, and the increment of nicotinamide was

Table 1. Stability of NAD and nicotinamide in fragments and homogenates prepared from normal dog heart*

Incubation at 37°C (min)	NAD	Nicotinamide
	(nmoles/g wet wt.)	
Cylindrical fragment		
0	349	112
15	339	158
30	308	178
60	328	155
Tissue homogenate		
0	450	130
5	13	565
15	33	541
30	14	618
60	0	626

*Values presented are from 1 animal. They are representative of a typical pattern found in all 8 animals in the normal group.

Table 2. Content and stability of NAD and nicotinamide in fragments of dog hearts with experimental myocardial infarction*

Dog	Incubation at 37°C (min)	Control Tissue		Infarcted Tissue	
		NAD	Nicotinamide	NAD	Nicotinamide
2 hr after occlusion					
1	0	351	105	245	189
	30	322	172	121	381
2	0	410	101	341	175
	30	303	193	123	305
24 hr after occlusion					
3	0	438	97	41	34
	30	340	204	25	37
4	0	401	114	94	86
	30	306	204	87	100

*Figures (nmoles/g wet wt.) are average values from separated analysis of 2 tissue samples.

almost equivalent to the loss of NAD. Furthermore, the amount of NAD originally present in the infarcted tissue decreased from 50 to 68%, and the level of nicotinamide increased stoichiometrically after 30 min of incubation at 37°C (Table 2). On the other hand, in the infarcted cardiac tissue removed 24 hours after the arterial occlusion, the levels of NAD and nicotinamide were very low and did not change after incubation at 37°C (Table 2).

The control tissue taken from hearts with coronary artery branch occluded behaved as slightly disorganized tissue in that part of the endogenous NAD was hydrolyzed during incubation of a fragment of this tissue, whereas the changes observed in normal cardiac tissue under the same conditions were smaller (Table 2).

NAD glycohydrolase activity in control and infarcted cardiac tissue

The NADase activity measured in homogenates was of the same order of magnitude in both the control and the infarcted cardiac tissue, irrespective of the time elapsed after the occlusion of the artery. The amount of nicotinamide released during the reaction was approximately equivalent to the NAD loss (Table 3).

Table 3. NAD glycohydrolase activity in homogenates from dog hearts with experimental infarction

Dog	Cardiac Tissue Sample	Enzyme Activity (μmoles/g wet wt., 15 min) NAD Consumed	Nicotinamide Produced
2 hr after coronary occlusion			
1	Control	15	17
	Infarcted	11	15
2	Control	9.4	12
	Infarcted	9.6	13
24 hr after coronary occlusion			
3	Control	12	10
	Infarcted	12	12
4	Control	11	9.8
	Infarcted	12	11

Table 4. Distribution of NAD glycohydrolase and marker enzymes in subcellular fractions from dog heart*

Fraction	Specific Activities (units/mg of protein)				
	NADase	Acid phosphatase	K^+-activated phosphatase	Na^+-K^+-Mg^{++} ATPase	Na^+-K^+ ATPase
Homogenate	43	64	62	2.9	0.9
Supernatant 1	19	64	46	1.5	0
Mitochondria	38	77	36	7.6	0.3
17,000 × *g*	515	327	266	27.3	7.0
105,000 × *g*	151	165	106	8.8	2.9

*Units: NADase, nmoles NAD hydrolyzed/mg, 15 min; acid phosphatase, nmoles *p*-nitrophenol hydrolyzed/mg, 15 min; K^+-activated phosphatase, nmoles *p*-nitrophenol hydrolyzed/mg, 10 min; ATPase, μmoles P_i/mg, hour.

Distribution of the NADase in the subcellular fractions of dog cardiac tissue

Specific activities in subcellular fractions for NAD glycohydrolase and for marker enzymes are given in Table 4. No activity of NADase was found in the final supernatant. The values for cytochrome *c* oxidase (change of absorbance at 550 nm/mg, min) were 18.6 in the mitochondrial fraction and 2.0 in the 17,000 × *g* fraction. This last fraction contained 44% of the total NADase activity, and around 27% appeared in the mitochondrial fractions. The original activity was almost recovered (80% in all the subcellular fractions separated from supernatant 1 (Table 5).

DISCUSSION

The unfavorable conditions, namely anoxia and/or ischemia, under which a fragment of normal heart was maintained *in vitro* for 1 hour, were not noxious enough to stimulate the degradation of the endogenous NAD, whereas the disorganization of the cardiac tissue appeared to determine the conditions for a reaction between an enzyme and NAD contained in the tissue.

We concluded that the enzyme hydrolyzes the NAD at the *N*-glycosyl bond between the pyridine and the ribose moieties since, both in the homogenate from normal hearts and in the fragment of infarcted tissue excised 2 hours after the arterial occlusion, the increment of nicotinamide was equivalent to the loss of NAD. Consequently, this enzyme seems to be analogous to that detected previously in hearts from other animal species (Spaulding and Graham, 1947;

Table 5. Recovery of NAD glycohydrolase in subcellular fractions from dog heart

Fraction	Total Volume (ml)	Units/ml	Total Kilounits	Distribution (%)
Homogenate	665	343	228	—
Supernatant 1	526	58	30.5	100
Light mitochondria	21	253	5.3	17.3
Heavy mitochondria	10	300	3.0	9.8
17,000 × *g*	10	1340	13.4	44
105,000 × *g*	7.5	378	2.8	9
Final supernatant	476	0	0	0

Govier and Jetter, 1948; Hulsmans, 1961; Waravdekar and Griffin, 1964; Grigorovich, 1970) and known as NAD glycohydrolase.

It is possible that the nicotinamide liberated by the NADase activity could be converted to other compounds and/or to escape toward the bloodstream. This would explain the very low levels of this compound found in the infarcted tissue 24 hours after occlusion.

The NADase activity appears to be localized mainly in a membranous fraction which we are trying to characterize. This 17,000 × *g* fraction was vesicular when examined with the electron microscope (Fig. 2) and disclosed the maximal specific activities of those enzymes which have been found in the plasma membrane fraction prepared from hearts (Kidwai *et al.*, 1971b) or myometrium of rats (Kidwai *et al.*, 1971a). Moreover, the NADase seems to be tightly bound the membranes, since the activity measured in the greatly disorganized infarcted tissue (Calva *et al.*, 1969) was as high as in the normal myocardium. In contrast to this finding, a significant rise in the activity of the hexosemonophosphate shunt enzymes and a rapid decline in the oxidative and glycolytic enzymes in the infarcted cardiac tissue have been reported (Gudbjarnason *et al.*, 1967).

The fact that the NAD glycohydrolase does not hydrolyze the endogenous NAD in the normal cardiac tissue could be explained by the topological separation between the NAD and the NADase. On the other hand, it has been established that the NADase has low activity on enzyme-bound NAD, and it has been assumed that most of the nicotinamide nucleotides exist in the cell bound to protein (Kaplan, 1966). Consequently, it is possible that, during the infarc-

tion process or the *in vitro* disruption of the cardiac tissue, the enzyme-bound NAD is converted to unbound coenzyme. Finally, although alterations in lysosome stability have been proposed to be among the earliest structural modifications to occur during the ischemic myocardial injury (Ricciuti, 1972), further work is necessary to elucidate the role of these cell structures in the hydrolysis of the NAD.

In a previous work, we postulated (Calva *et al.*, 1966) that the blockade of the respiratory chain observed in mitochondria from infarcted cardiac tissue could be due to the loss of mitochondrial NAD. We can now add that it is possible this loss may be due to the activity of the NADase as a result of the tissue disintegration. It has not been determined whether such activity is also present

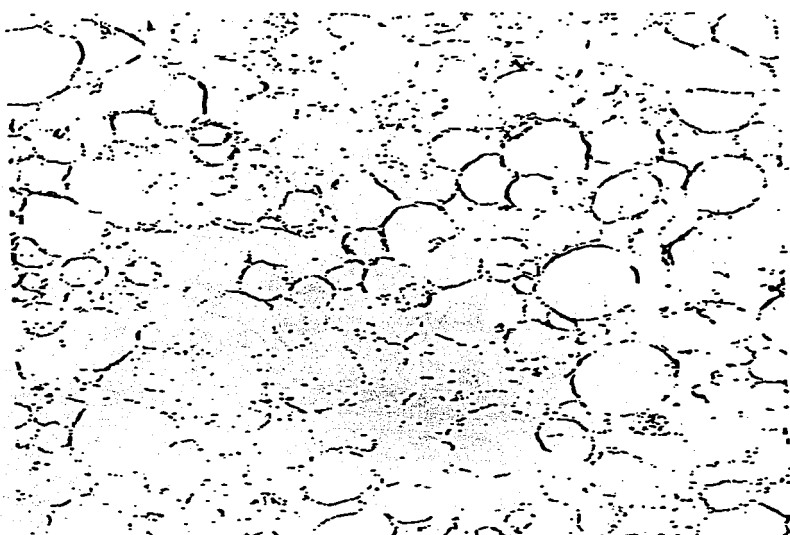

Figure 2. Electron micrograph of thin section prepared from the 17,000 × *g* fraction of dog heart homogenates. × 48,000. Courtesy of M. González del Pliego, Laboratory of electron microscopy, Inc.

in the cardiac mitochondria or whether it is due to a membranous contamination of the mitochondrial fraction.

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