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ASPECTOS CINETICOS Y ESTRUCTURALES DE DOS 17β-HIDROXIESTEROIDE DESHIDROGENASAS DE ORIGEN ANIMAL

T E S I S

Que para obtener el Grado de :

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P r e s e n t a:

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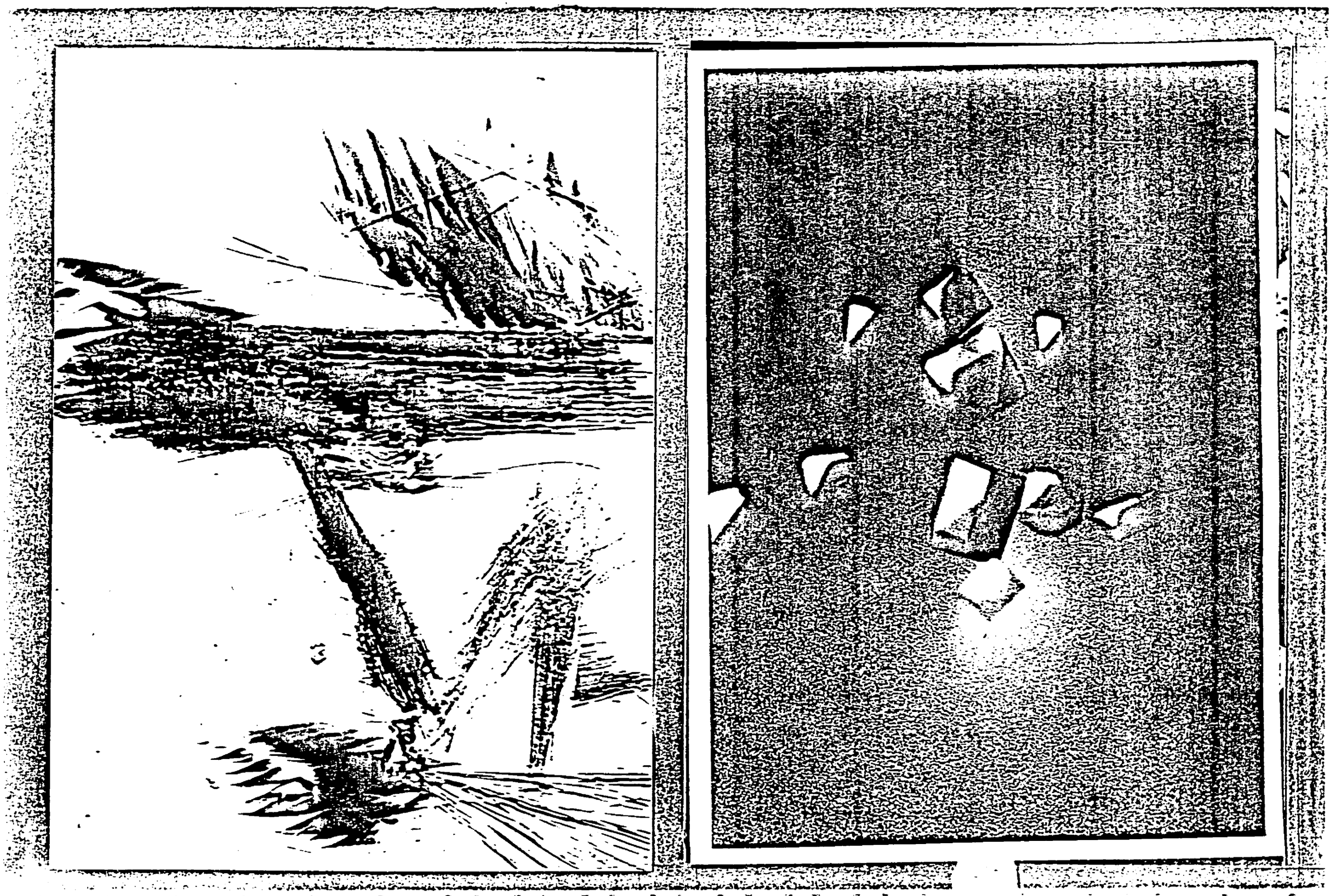

17β-ESTRADIOL DESHIDROGENASA DE PLACENTA HUMANA: Inhibición por el derivado 2',3'-dialdehído del $NADP^+$.

ANTECEDENTES:

Caracterizada inicialmente en 1958 (1), la 17β-estradiol deshidrogenasa (EC 1.1.1.62) del citosol de placenta humana es la enzima del metabolismo de hormonas esteroides más ampliamente estudiada en tejidos animales y objeto central de interés para muchos grupos de investigación. Es hasta el momento, la única de las enzimas transformadoras de hormonas esteroides que se ha logrado aislar en forma pura de tejidos humanos, y en cantidades que permiten abordar un estudio químico detallado.

Cataliza la oxidación reversible de 17β-estradiol a estrona utilizando NAD^+ o $NADP^+$ como cosubstratos (1,2). Cataliza también la reducción, aparentemente irreversible, de 16α-hidroxiestrona a estriol (3), siendo este último el estrógeno más abundante en el embarazo humano; la 17β-oxido-reducción de varios andrógenos (4-5) y la reducción en posición 20α de la progesterona (5-7).

Aunque su papel fisiológico no se ha establecido, resulta evidente que una enzima presente en el citosol de la placenta humana que cataliza oxido-reducción de estrógenos, andrógenos y progesterona, es un candidato potencial a tener una participación importante en la regulación hormonal en el ambiente materno y feto placentario.

La 17β-estradiol deshidrogenasa tiene un peso molecular de 68,000 y esta constituída por dos cadenas polipéptidicas de idéntico peso molecular (33,500-35,000). Cuando la enzima se somete a enfoque isoeléctrico en gel de poliacrilamida en presencia de urea 8M, se disocia en tres bandas con distinta concentración de proteína. Estos resultados son congruentes con la presencia de cantidades desiguales de tres monómeros que teniendo el mismo peso molecular poseen una carga neta distinta, pudiendo interactuar para formar seis dímeros. Heterogeneidad atribuible a la presencia de isómeros de carga se ha reportado también en la 17β-hidroxiesteroide deshidrogenasa purificada de testículo de cerdo, la que se ha descrito como una enzima monomérica con peso molecular de 35,000 (8).

El análisis de aminoácidos de la 17β-estradiol deshidrogenasa se ha llevado a cabo por diferentes grupos (norteamericanos y franceses), y recientemente por nosotros. Como se aprecia en la Tabla I, se encuentran diferencias notables en los resultados obtenidos por cada grupo. Aún cuando no hay una explicación satisfactoria para estas discrepancias, se ha sugerido que pueden atribuirse al hecho de que la enzima se obtiene de placentas provenientes de poblaciones genéticamente diferentes (3).

En el análisis N-terminal, realizado por Burns *et al.* (9), utilizando el procedimiento de Sanger, demuestra un solo aminoácido N-terminal: alanina. Una secuencia N-terminal de cinco residuos de aminoácidos obtenida mediante la degrada-

TABLA I

17β-Estradiol Deshidrogenasa: Composición de Aminoácidos

AMINOACIDO	Residuos/dímero USA (*1)	FRANCIA (2)	MEXICO (3)
Lys	20	30	14
His	14	14	12
Arg	44	34-36	36
Asp	42	54-56	34
Thr	32	32-34	24
Ser	38	40	26
Glu	56	68-70	46
Pro	38	40	38
Gly	62	48	58
Ala	72	54	66
½Cys	12	12	12
Val	64	52	80
Met	8	12	12
Ile	8	20	8
Leu	82	66	100
Tyr	12	16	10
Phe	26	24	22
Trp	2	4	2

(1) Burns, D.J.W., Engel, L.L., and Bethune, J.L. (1972) Biochemistry 11, 2699-2703.

(2) Nicolas, J.C., Pons, M., Descomps, B., and Crastes de Paulet, A. (1972) FEBS. Lett. 23, 175-179.

(3) La composición de Aminoácidos fue amablemente realizada por el Dr. Lourival Possani. Los resultados son producto de seis análisis.

-ción de Edman por los mismos autores, confirma la presencia de alanina en el extremo N-terminal: Ala-Glu-Thr-Val-Val-(10). El Dr. Lourival Possani amablemente realizó la degradación de Edman de la enzima purificada en nuestro laboratorio, determinando la secuencia N-terminal de trece residuos de aminoácidos Ala-Arg-Thr-Val-Val-Leu-Ile-Thr-Val-Cys-Ser-Arg-Gly-. A pesar de la discrepancia respecto al segundo aminoácido, se identificó un único péptido N-terminal en ambas secuencias. En tanto que estos resultados sugieren que ambas subunidades en la estradiol deshidrogenasa son idénticas, no descartan la posibilidad de que el amino terminal de una subunidad se encuentre bloqueado o inaccesible, o bien que las subunidades difieran en aminoácidos más internos.

Actualmente y gracias a la disponibilidad de cantidades suficientes de enzima, estamos llevando a cabo estudios para determinar la estructura primaria de la 17β-estradiol-deshidrogenasa. Enzima reducida y alquilada con ditiotreitol y 4-Vinilpiridina por el método de Possani *et.al.*[(1981) - Carlsberg Res.Commun. 46, 195-205],se envió, liofilizada, al Dr. Brian M. Martin quién realizó la ruptura tríptica, separó los péptidos por HPLC y determinó la secuencia de aminoácidos de algunos de ellos. (Tabla II).

Estudios realizados sobre el mecanismo de reacción de la 17β-estradiol deshidrogenasa empleando el método de in-

T A B L A II

SECUENCIA DE 5 PEPTIDOS PRODUCTO DE LA RUPTURA CON TRIPSINA DE LA 17β ESTRADIOL DESHIDROGENASA

Glu-Val-Phe-Gly-Asp-Val-Pro-Ala-Lys-Ala-Glu-Ala-Gly-Ala-Glu-Ala-Gly-Gly-Gly-Ala-Gly-Pro.

Phe-Tyr-Gln-Tyr-Leu-Ala-His-Ser-Lys.

Ala-Pro-Lys-Pro-Thr-Leu-Arg.

Ala-Leu-Ala-Cys-Pro-Pro-Gly-Ser-Leu-Glu-Thr-Leu-Glu-Leu-Asp.

Phe-Leu-Pro-Leu-Leu-Arg.

tercambio isotópico en equilibrio, demuestran que tanto el esteroide como el nucleótido forman complejos binarios con la enzima, estos resultados son congruentes con un mecanismo al azar (11). La estereoespecificidad de la transferencia del hidruro del nucleótido reducido al substrato esteroidal también se ha establecido; la enzima cataliza la transferencia del hidrógeno 4-pro-S del NADH o del NADPH a la posición 17α del esteroide (12).

La muy amplia substrato-especificidad de la 17β-estradiol deshidrogenasa, el carácter relativamente rígido de los esteroides, la estricta estereoespecificidad en la transferencia del hidruro de los cosubstratos al esteroide, la existencia de procedimientos cada vez más simples y con mayor rendimiento para la purificación de esta enzima (13-15), y la relativa sencillez en la cuantificación de su actividad, son características que hacen de la 17β-estradiol deshidrogenasa una de las moléculas más adecuadas para el estudio de las interacciones de las proteínas con esteroides y nucleótidos mediante las técnicas de marcado de afinidad.

Se han sintetizado una variedad de análogos esteroidales, substratos o inhibidores de la 17β-estradiol deshidrogenasa, portadores de grupos reactivos en diferentes posiciones del núcleo esteroidal. Estos reactivos pueden formar uniones covalentes con los residuos de aminoácidos en la proteína. Utilizando estos análogos ha sido posible identificar varios

de los aminoácidos localizados en el sitio de unión de esteroides de la estradiol deshidrogenasa, estableciendo su posición relativa respecto a los anillos del esteroide (16-21).

En el transcurso de estos estudios se ha observado que los nucleótidos de piridina modifican la cinética de inactivación de la 17β-estradiol deshidrogenasa por los marcadores esteroidales. Estos resultados sugieren que algunos marcadores posiblemente se unen a la región hidrofóbica de la enzima normalmente ocupada por la porción de la adenina de los nucleótidos de piridina; sin embargo, cuando se comparan, los cambios ejercidos por el NAD^+ son marcadamente diferentes a los observados en presencia de $NADP^+$ (3,22-23).

En base a las consideraciones anteriores y con el propósito de avanzar en la caracterización del sitio activo de la 17β-estradiol deshidrogenasa de placenta humana, surge el interés de buscar compuestos que puedan ser utilizados como marcadores dirigidos a la región de unión para nucleótidos en el sitio activo de esta enzima.

En el siguiente trabajo se reportan los efectos del derivado 2',3'-dialdehído del $NADP^+$ sobre la 17β-estradiol-deshidrogenasa del citosol de placenta humana.

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PERIODATE-OXIDIZED NADP$^+$ IS A POWERFUL INHIBITOR OF HUMAN PLACENTAL ESTRADIOL-17β DEHYDROGENASE

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Summary: Periodate-oxidized NADP$^+$ inhibits the NAD$^+$-linked activity of human placental estradiol-17β dehydrogenase (EC 1.1.1.62). The inhibition appears to be competitive with respect to NAD$^+$ and can be reversed by dialysis or gel filtration. The apparent inhibitor constant for the periodate-oxidized analogue is 0.047 μM. The presence in the incubation mixture of NAD$^+$ protects the enzyme against inhibition. No inhibitory effects of the coenzyme analogue are observed on the NADP$^+$-linked activity of the enzyme.

Estradiol-17β dehydrogenase (EC 1.1.1.62) from human term placenta is a dimeric enzyme that has a rather broad steroid substrate specificity, catalyzing the reversible interconversion of estradiol-17β and estrone, the apparent irreversible reduction of 16α-hydroxyestrone to estriol and the oxidation-reduction of several androgens and progesterone (1,2). The enzyme utilizes both NAD$^+$ and NADP$^+$ as cosubstrates (3); NADP$^+$ is more tightly bound to the enzyme than NAD$^+$ (4). 3-acetylpyridine adenine dinucleotide and certain NAD(P)$^+$ analogues obtained by replacing substituents in the nicotinamide or adenine rings are also efficient hydrogen acceptors (5,6).

The topography of the steroid-binding site has been studied by affinity labeling techniques using a different series

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affinity step, by gel filtration on Sephadex G-25, equilibrated in 5 mM phosphate buffer, pH 7.1, containing 1 mM EDTA and 20% glycerol. The enzyme solution was concentrated by ultrafiltration (Amicon Diaflo ultrafilter PM-10). The homogeneity of the purified enzyme was routinely checked by polyacrylamide-gel electrophoresis under denaturing conditions, using the discontinuous buffer system of Laemmli (15).

Protein concentrations were determined by the Coomassie blue G-binding method of Bradford (16).

Assay Method. Enzyme assays were done by spectrophotometric measurement of the reduction of NAD^+ or $NADP^+$. Reaction velocities were calculated from the initial linear increase in absorbance at 340 nm in a single beam automatic recording spectrophotometer (Gilford model 252). Estradiol-17β was the only steroidal substrate used. The reaction cuvettes contained 0.23 µmole of NAD^+ or 0.19 µmole of $NADP^+$, 0.05 µmole of estradiol-17β (added in 10 µl of 95% ethanol), in 5 mM phosphate buffer, containing 20% glycerol with a total volume of 0.5 ml, pH 7.1. The assays were conducted at room temperature and initiated by addition of appropriate quantities of the enzyme.

The assay system for the determination of the kinetics constants was identical with that already described, but substrates and inhibitor were added in varying concentrations.

To evaluate the effect of the presence of substrates on the inhibition by periodate-oxidized analogue, the enzyme (1.0 nmol of subunit/ml) was incubated with different concentrations (from 0.01 to 0.4 µM) of cosubstrate analogue in presence or absence of either 2.5 mM NAD^+ or 0.1 mM estradiol-17β and assayed for residual catalytic activity; reactivation on dilution in the assay cuvettes was not detectable.

The reversibility of the inactivation was investigated in the following experiments: The enzyme (1.0 nmol of subunit/ml) was treated with periodate-oxidized $NADP^+$ (final concentration: 0.1 µM), aliquots were removed, tested for inhibition and then subjected to gel filtration on Sephadex G-25 equilibrated in buffer as described above, and assayed for catalytic activity.

The Km and Vmax values of the inhibition series were estimated according to Wilkinson (17). The slope replot was made with these calculated parameters and the Ki was obtained by means of linear least-squares.

RESULTS AND DISCUSSION

Periodate-oxidized $NADP^+$, a cosubstrate analogue with aldehyde groups at the 2'- and 3'- positions of the ribose ring bound to the nicotinamide, was tested for activity or inhibition of human placental estradiol-17β dehydrogenase. The oxidized analogue appears to be a powerful inhibitor of the NAD^+-linked activity of the enzyme. The inhibition shows a classical competitive pattern with respect to NAD^+ (Fig. 1). The in

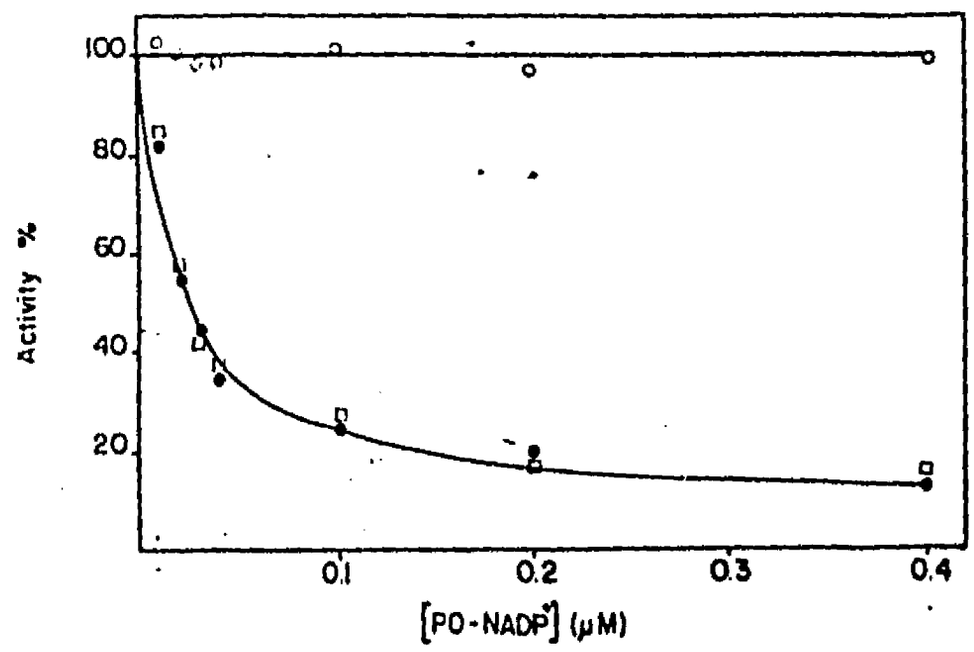

Fig.2. Effects of the presence of NAD^+ and of estradiol-17β on the inhibition by periodate-oxidized $NADP^+$. The enzyme was incubated with the inhibitor at the concentrations indicated in the abscissa; •, no further additions; □, in the presence of 0.1 mM of estradiol-17β; o, in the presence of 2.5 mM of NAD^+.

17β has no effect, the presence of NAD^+ in the incubation mixture fully protects the enzyme against inhibition. These results suggest that the inhibitor is bound specifically at the cosubstrate-binding site of the enzyme.

Estradiol-17β dehydrogenase 75.5% inactivated by treatment with the oxidized analogue recovers its initial enzymatic activity by gel filtration or prolonged dialysis, showing the reversible nature of inhibition and excluding the possibility that the inhibition could be due to denaturation of the enzyme.

Inactivation of estradiol-17β dehydrogenase by periodate-oxidized $NADP^+$ shows to be unusually effective with almost instantaneous formation of the enzyme-inhibitor complex and thus the time course of inhibition could not be obtained in the assay conditions described in this paper.

No inhibitory effect of the periodate-oxidized analogue at the highest concentration used (0.2 mM) was observed on the $NADP^+$-linked activity of the enzyme. A similar behavior has

also been found with other NAD(P)$^+$ analogues (5). Engel et al. studing a series of adenine nucleotides, reported that 2'-adenosine monophosphate, the adenosine moiety of NADP$^+$, at a concentration of 0.5 mM inhibits the NAD$^+$-linked activity of the enzyme, but no inhibitory effects on the NADP$^+$-linked activity were observed (18). It has been suggested that the failure of the tested compounds to inhibit the NADP$^+$-linked activity, as well as the different behavior observed between NAD$^+$ and NADP$^+$ on enzyme inhibition upon steroid affinity labels or NAD(P)$^+$ analogues (5-7,19), could be a consequence of the relatively high values of the corresponding inhibition constant and the different binding affinities for the two pyridine nucleotides. As described above, NADP$^+$ has more affinity for the enzyme than NAD$^+$. Periodate-oxidized NADP$^+$ inhibits specifically the NAD$^+$-linked activity of the enzyme, competing with NAD$^+$. The inhibition constant for this analogue is 1000 times smaller than the Km value for NAD$^+$ (53 μM) and about one twentieth of that for NADP$^+$ (0.89 μM). These results suggest that the two pyridine nucleotides could have different binding sites on estradiol-17β dehydrogenase.

Further studies to provide direct evidence that periodate-oxidized NADP$^+$ is bound at the site occupied by the cosubstrate are needed. Since periodate-oxidized NADP$^+$ is the best competitive inhibitor so far reported, we belive that this reagent provides a useful tool for studies on the active site of the enzyme.

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COMENTARIOS:

Varias enzimas $NAD(P)^+$-dependientes contienen residuos de lisina en el dominio de unión para el cosubstrato (24,25). El tratamiento del $NADP^+$ con *m*-peryodato ($NaIO_4$) produce un derivado con grupos aldehído en los carbonos 2',3' del anillo de ribosa unida a la nicotinamida (26). Este derivado tiene la posibilidad de reaccionar con los grupos ε-amino de residuos de lisina en las proteínas.

Dallocchio *et al.* en 1976 demostraron la naturaleza sitio-específica de la inhibición producida por el derivado dialdehídico del $NADP^+$ (oNADP) sobre la 6-fosfogluconato deshidrogenasa de *Candida utilis* (27). A partir de este reporte el oNADP y análogos dialdehídicos de otros nucleótidos (oATP, oNAD, oADP, 8-azido-oATP) han demostrado su utilidad como marcadores de afinidad de enzimas que utilizan nucleótidos como substratos o efectores alostéricos.

En este estudio, el análogo 2',3'-dialdehídico del $NADP^+$ se preparó por oxidación con peryodato y de acuerdo con los siguientes criterios se considera un marcador de afinidad del sitio de unión para nucleótidos de la 17β-estradiol deshidrogenasa de placenta humana.

El análogo del cosubstrato mostró ser un inhibidor potente de la actividad dependiente de NAD^+ de la 17β-estradiol

deshidrogenasa.

La inhibición muestra un comportamiento competitivo clásico con respecto al cosubstrato natural: NAD^+.

La naturaleza reversible de la inhibición indica que la interacción con el análogo no produce daño de la proteína y sugiere la existencia de un equilibrio proteína libre, proteína inhibidor.

La presencia de concentraciones saturantes de NAD^+ en la mezcla de incubación brindan una protección completa en contra de la inactivación ocasionada por el análogo del cosubstrato, en tanto que, la presencia de concentraciones saturantes del substrato esteroidal (17β-estradiol), no modifican la cinética de inactivación.

La constante de inhibición nanomolar obtenida para el análogo, puede explicarse por la formación de una aldimina entre el aldehído 2' o 3' del oNADP y un grupo amino en el sitio activo de la enzima.

Experimentos adicionales señalan que la inhibición competitiva y reversible producida por el análogo oxidado con peryodato, se transforma en una inactivación irreversible por tratamiento con $NaBH_4$. Estos resultados sugieren que, en efecto, a la formación del complejo binario enzima-oNADP se sigue la reacción con un grupo amino en, o cerca del sitio activo de la 17β-estradiol deshidrogenasa para producir una aldimina, la cual se reduce a una amina secundaria por acción del borohidruro.

Una evidencia directa de la especificidad de la reacción entre la 17β-estradiol deshidrogenasa y el derivado oxidado del $NADP^+$ deberá proporcionarse identificando el, o los residuos de aminoácidos modificados, presumiblemante Lys-oNADP. Experimentos en este sentido se están llevando a cabo.

Aún quedan muchos problemas por resolver respecto a la función, estructura y regulación de la 17β-estradiol deshidrogenasa: ¿Cuáles son las interacciones que permiten que un mismo sitio activo pueda unir esteroides C_{18}, C_{19} y C_{21}? ¿Cuáles son las disposiciones espaciales entre los substratos y la proteína que permiten que dos actividades (17β- y 20α-hidroxiesteroide deshidrogenasa) compartan un mismo sitio? ¿Cómo se regulan ambas actividades?. La respuesta a estas interrogantes requiere generar información acerca de la estructura primaria y de la geometría de las cadenas polipeptídicas que comprenden el sitio activo y dilucidar el papel que juegan los distintos aminoácidos en los procesos de unión y catálisis.

En tanto no sea posible llevar a cabo estudios por difracción de rayos-X, que porporcionen información de la estructura terciaria y de la flexibilidad que permite que ambas moléculas esteroidales compartan un mismo sitio activo en la 17β-estradiol deshidrogenasa, las técnicas de "marcaje de afinidad" seguirán siendo la alternativa más útil en el estudio de las interacciones de la proteína con esteroides y nucleótidos.

17β-HIDROXIESTEROIDE DESHIDROGENASA DE PANCREAS CANINO: Descripción, Ensayo y Caracterización.

ANTECEDENTES:

Aunque previamente no se había considerado, un número creciente de evidencias permiten ahora suponer que el páncreas tiene un papel importante en la biosíntesis y metabolismo de las hormonas esteroides.

La primera observación en este sentido, fué hecha en 1963 por Ullberg y Bengtsson (28), quienes llevando a cabo un estudio cualitativo mediante autoradiografías de la distribución de estrógenos en tejidos de ratones, reportan que el páncreas capta y retiene una concentración relativamente alta de radiactividad varias horas después de la administración de estrógenos marcados. No obstante su importancia, esta observación permaneció olvidada durante casi una década.

La segunda notificación a este respecto, aparece en 1972; cuando Kirdani *et al.* (29), reportan estudios sobre la distribución en los tejidos de estrógenos y andrógenos diferentemente marcados, después de su administración simultánea intravenosa a perros mestizos y babuinos. Biopsias de distintos tejidos del mismo animal se tomaron a intervalos de quince y treinta minutos, durante un período de tres horas. Más radioactividad y durante mayor tiempo se retuvo en el páncreas de ambas especies de animales que en cualquier otro órgano, incluyendo tejidos

esteroidogénicos. Resultados similares se obtuvieron en la rata, puerco de guinea, perro y babuino después de la administración de estradiol, estriol o dietilestilbestrol.

Reportes posteriores demuestran la presencia de receptores para estrógenos y andrógenos en las células acinares del páncreas de distintas especies de mamíferos, incluyendo la especie humana (30-34).

Por otra parte, datos epidemiológicos señalan que la incidencia de carcinoma de páncreas es significativamente más elevada en hombres que en mujeres (35-36).

Alteraciones en el perfil sérico de andrógenos, consistentemente niveles bajos de testosterona, se han reportado en pacientes afectados de carcinoma de páncreas (37-38).

En la carcinogénesis experimental inducida por fármacos (4-hidroxiaminoquinoleina 1-oxido y azaserina), se demostró que la incidencia y el crecimiento de tumores de páncreas son más elevados en las ratas macho que en las hembras. En estos estudios también se señala que la administración de la droga seguida de la castración, disminuye la incidencia de estos tumores en ratas macho, en tanto que la castración seguida de la administración de testosterona, resulta en una elevada incidencia de tumores en animales de ambos sexos (39-40).

Estudios comparativos señalan niveles altos de receptores para estrógenos en las fracciones citosólica y nuclear de tejido pancreático humano tumoral y fetal, en relación con el páncreas normal adulto (41).

Finalmente, las actividades de Aromatasa y 5α-reductasa, enzimas que participan en la biosíntesis de hormonas esteroides, se determinaron en homogenados de tejido pancreático humano, encontrando diferencias cuantitativas entre carcinoma, tejido fetal y páncreas normal adulto (42).

Los reportes anteriores además de llamar la atención sobre la importancia que puede tener el metabolismo de hormonas esteroides en el páncreas, sugieren también la naturaleza "hormona-dependiente" del carcinoma de esta glándula, lo que implica que el manejo hormonal puede tener valor terapéutico.

En base a las consideraciones señaladas, tenemos interés de estudiar las reacciones enzimáticas y establecer las vías de síntesis y transformación de hormonas esteroides que tienen lugar en el páncreas.

Aquí se reporta la caracterización en páncreas canino de una 17β-hidroxiesteroide deshidrogenasa que cataliza la interconversión de androstendiona y testosterona.

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17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN CANINE PANCREAS

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Summary: The mitochondrial fraction of the dog pancreas showed NAD(H)-dependent enzyme activity of 17β-hydroxysteroid dehydrogenase. The enzyme catalyzes oxidoreduction between androstenedione and testosterone. The apparent Km value of the enzyme for androstenedione was 9.5 ± 0.9 μM, the apparent Vmax was determined as 0.4 nmol mg^{-1} min^{-1}, and the optimal pH was 6.5. In phosphate buffer, pH 7.0, maximal rate of androstenedione reduction was observed at 37°C. The oxidation of testosterone by the enzyme proceeded at the same rate as the reduction of the androstenedione at a pH of 6.8-7.0. The apparent Km value and the optimal pH of the enzyme for testosterone were 3.5 ± 0.5 μM and 7.5, respectively.

Over the last few years a number of interesting observations have provided evidence suggesting the involvement of the pancreas in the biosynthesis and metabolism of steroid hormones. Estrogen binding globulins and steroid receptors have been reported in normal and malignant pancreatic tissue (1-4). Aromatase and 5α-reductase activity has been measured in cell-free homogenates of human pancreatic tissue (5). Several changes in the serum androgen profile, mainly low testosterone levels, have been found to be related to human pancreatic carcinoma (6-8), and recent studies on experimental pancreatic carcinoma in rat suggested that this tumor could be hormone-responsive (9,10). Despite the importance of steroid metabolism in the pancreas, few studies have been performed and little information on the pancreas ability to synthesize steroids is available.

To obtain more information on the steroidal functions of the pancreas, it appeared of interest to study the enzymatic activ-

Abbreviations: EDTA, ethylene diamine N,N,N',N'-tetraacetic acid; PMSF, phenyl methylsulfonyl fluoride; (SE), standard error; androstenedione, androst-4-ene-3,17-dione; testosterone, 17β-hydroxyandrost-4-en-3-one.

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ities involved in the metabolic transformations of steroid hormones in this gland. The present paper reports an active 17β-hydroxysteroid dehydrogenase in the mitochondrial fraction of the dog pancreas, which catalyzes oxidoreduction between androstenedione and testosterone. This finding supports the hypothesis that the pancreas could be an extragonadal site of steroid hormone metabolism.

MATERIALS AND METHODS

Reagents. [1,2-^{3}H]Androstenedione (specific activity, 46.1 Ci/mmol) and [1,2-^{3}H]testosterone (specific activity, 49.0 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.), and rechromatographed on a Sephadex LH-20 column, equilibrated and developed with the solvent system toluene:methanol (85:15, v/v). The radioactive purity of the steroids was confirmed by thin-layer chromatography in a system of methylene dichloride: ethyl acetate (80:20, v/v) prior to use. The radioactive steroids were diluted with the corresponding authentic non-radioactive steroids purchased from Steraloids Inc. (Wilton, N.H.) to obtain appropriate specific radioactivity for the enzyme assays.

Pyridine nucleotides (NAD^+, NADH, $NADP^+$, NADPH), PMSF and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, Mo.), thin-layer chromatography sheets and analytical-grade solvents were obtained from E. Merck (Darmstadt, Germany). All the chemical reagents used were of analytical grade. Deionized water was used for all aqueous solutions.

Tissue preparation. Pancreas of male mongrel dogs were obtained from the Instituto Nacional de Cardiología (México, D. F.). After transportation in ice to the laboratory, tissues were dissected away from obvious fat and connective tissue, weighed, minced with scissors, and homogenized in ice-cold 0.25 M sucrose solution containing 0.05 M potassium phosphate buffer, 1 mM EDTA, 5 mM $MgCl_2$, 10 μM PMSF, and 7 mM 2-mercaptoethanol, pH 7.0, with a weight/volume ratio of 1:10. Homogenization was performed in a Polytron apparatus with three 20 sec bursts at 3000 rpm. Subcellular fractions were prepared by differential centrifugation (11). All operations were conducted at 0-4°C. The homogenate was subjected to a 600 x g, 10-min centrifugation to eliminate nuclei, unbroken cells, and heavy debris. The resulting supernatant was centrifuged at 1000 x g for 10 min to sediment the zymogen granules. The mitochondrial fraction was sedimented by centrifugation for 15 min at 8700 x g. The pellet was washed three times with homogenization buffer and sedimented at 12,000 x g. The initial supernatant from the mitochondrial fraction was then centrifuged at 109,000 x g for 60 min, the resulting pellet and supernatant were employed conventionally as the microsomal and cytosol fractions of dog pancreas, respectively. The subcellular fractions were kept at -20°C until incubations were started.

Enzyme assays. 17β-hydroxysteroid dehydrogenase was determined by measuring both the reduction of androstenedione to testosterone and androstenedione formation from testosterone. Unless otherwise noted, the assay mixture contained, in a total volume of 1 ml, steroid substrate (50 nmol; 200,000 cpm) added in 0.05 ml

of 95% ethanol, 1.35 μmol of NAD^+ or 1.35 μmol of NADH, dissolved in 5 mM potassium phosphate buffer, containing 1 mM EDTA, 5 mM $MgCl_2$, 10 μM PMSF, and 7 mM 2-mercaptoethanol, pH 7.0. The reactions were initiated by the addition of appropriate quantities of the enzyme preparation. The assays were conducted at 37°C for 10 min in air under constant shaking in a Dubnoff-type metabolic incubator. Blank incubations were prepared without nucleotide cosubstrate as well as using enzyme preparations inactivated by heating at 98°C for 2 min.

Enzyme reactions were stopped by the addition of 10 volumes of diethyl ether; androstenedione and testosterone (50 μg each per flask) were added to the mixture as carrier steroids, and the extraction was repeated twice with 10 volumes of diethyl ether. The extracts were pooled and evaporated to dryness under reduced pressure. The final residue was redissolved in 0.2 ml of ethanol. An aliquot of 0.05 ml was used to quantitate recovery and 0.05 ml was chromatographed on a 0.2 mm thin-layer sheet of silica gel 60 F_{254}, in a solvent system of methylene dichloride:ethyl acetate (80:20, v/v). The spots of the carrier steroids were visualized under ultraviolet light at 254 nm. The silica gel containing the identified compounds was scraped out of the thin-layer sheet and transferred into scintillation vials.

For scintillation counting, 10 ml of a toluene solution, containing 0.5% 2,5-diphenyloxazole and 0.05% 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene, was added to each vial. Radioactivity was determined using a Packard Tri-carb Liquid Scintillation Spectrometer (Model 3255). The counting efficiency for tritium was of 60%.

The amount of product was expressed in nmoles by dividing its radioactivity by the specific activity of the initial substrate. 17β-hydroxysteroid dehydrogenase activity was expressed as nmoles of product formed per mg of protein per min.

Protein concentrations were determined by the Coomassie blue G dye-binding technique of Bradford (12), with bovine serum albumin used as standard.

Kinetic data were fitted to the Michaelis-Menten equation and the corresponding parameters calculated according to Wilkinson (13).

RESULTS AND DISCUSSION

Identification of Products. Only one radiolabeled product which comigrated with testosterone was formed from [^{3}H]androstenedione in incubation mixtures containing cell-free homogenates of dog pancreas and NADH. This compound was identified as authentic testosterone demonstrating constant specific radioactivity through recrystallization with non-radioactive testosterone. Similarly, the only radioactive product obtained from [^{3}H]testosterone in the presence of NAD^+ was identified as androstenedione by its mobility on thin-layer chromatography and recrystallization with authentic non-radioactive androstenedione. The radioactivity recovered as androstenedione and testosterone on the thin-layer chromatograms averaged 93% under the conditions used in the pres-

ent study. No detectable radioactive compounds were formed when heat-denatured homogenates of dog pancreas were employed.

Subcellular Localization. The results obtained on subcellular fractionation of the homogenate are summarized in Table I. More than 86% of both oxidative and reductive activities of the 17β-hydroxysteroid dehydrogenase, found in the whole homogenate, were concentrated in the mitochondrial fraction with a 4-fold increase in the specific activity. The remaining activity was found in the particulate sediment of centrifugation at 600 x g. Treatment of the mitochondrial fraction with phospholipase A resulted in a 90% decrease of the enzyme activity and attemps to solubilize the enzyme, using sonication as reported for the testicular microsomal 17β-hydroxysteroid dehydrogenase (14), were unsuccessful. These observations suggest that the enzyme is tightly bound to the membrane and its activity related to the membrane structure.

Cofactor Requirement. 17β-hydroxysteroid dehydrogenase of dog pancreas appeared to be a NAD(H)-specific enzyme. Androstenedione was not reduced by the enzyme when NADPH at the high concentration of 2.0 mM was used as cosubstrate. Similarly, $NADP^+$ could not substitute for NAD^+ in the reverse enzyme reaction for testosterone. At a high fixed concentration of the corresponding steroid substrate (80 μM), the apparent Michaelis constants (Km) for NADH and NAD^+ were determined to be 157 μM and 315 μM, respectively. Bioconversion of steroid substrates could not be detected in the absence of added pyridine nucleotide.

The mitochondrial localization and the NAD(H)-specificity of this enzyme are in contrast to the microsomal localization and

TABLE I

INTRACELLULAR DISTRIBUTION OF THE 17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN CANINE PANCREATIC TISSUE

Subcellular Fraction	Protein mg.ml	Volume ml	Androstenedione Reduction: Specific activity (nmol.min^{-1}mg^{-1})	Androstenedione Reduction: Total activity (μmol.min)	Androstenedione Reduction: Activity recovered (%)	Testosterone Oxidation: Specific activity (nmol.min^{-1}mg^{-1})	Testosterone Oxidation: Total activity (μmol.min)	Testosterone Oxidation: Activity recovered (%)
Homogenate	14.8	505.0	0.081	0.605	100.0	0.111	0.829	100.0
Nuclei	14.5	50.0	0.104	0.075	12.4	0.150	0.108	13.0
Mitochondria	37.8	45.0	0.317	0.539	89.0	0.420	0.714	86.1
Microsome	38.2	11.0	0.027	0.011	1.8	0.051	0.021	2.5
Cytosol	11.3	380.0	0.000	0.000	00.0	0.003	0.012	1.4

the NADP(H) of the testicular 17β-hydroxysteroid dehydrogenases (14-16).

Kinetics. The amount of either testosterone or androstenedione produced from the corresponding substrates by the mitochondrial fraction of dog pancreas increased proportionately to the protein concentrations of the tissue preparation (Fig.1a). In time-course studies of the reductase and oxidase functions of 17β-hydroxysteroid dehydrogenase with a fixed protein concentration of the pancreatic mitochondrial fraction, the amount of the respective steroid product increased linearly with time of incubation for at least 30 min (Fig.1b).

pH Dependency. The pH dependence of both, reductase and oxidase activities of the pancreatic enzyme was examined using three different buffer systems; 0.05 M sodium acetate, pH between 4.0 and 6.0; 0.05 M Tris-HCl, pH from 6.5 to 9.0; and 0.05 M glycine-NaOH, pH from 9.5 to 12.0. The optimal pH of the enzyme for androstenedione reduction was found to be at 6.0-6.5, whereas for oxidation of testosterone it was 7.5. The enzyme reaction was found to be reversible at a pH between 5.5 and 8.5, with oxidation and reduction ocurring at approximately the same rate at a pH of around 7.0 (Fig.2a).

Temperature Dependency. Initial velocities of the enzyme reaction were determined at an incubation temperature between 20°C and 70°C

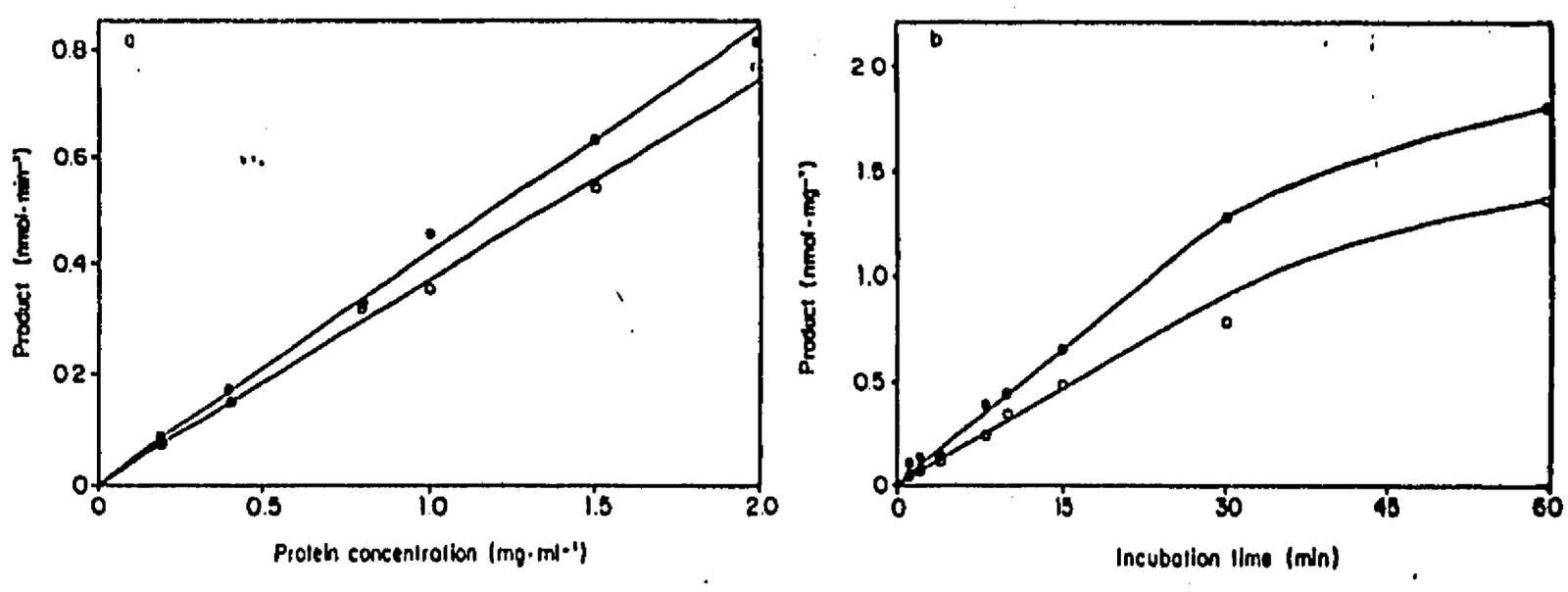

Fig.1.(a): *Effect of protein concentrations of the pancreatic tissue preparation on the product formation*. Enzyme activity was determined as described in the text. The amount of testosterone (o—o) or androstenedione (•—•) formed by the mitochondrial fraction was plotted against protein concentration.
(b): *Time-course of product formation with the pancreatic tissue preparation*. Incubations were performed with the mitochondrial fraction (1.0 mg of protein per flask) as described in the text, and the amount of testosterone (o—o) or androstenedione (•—•) formed was plotted against incubation time.

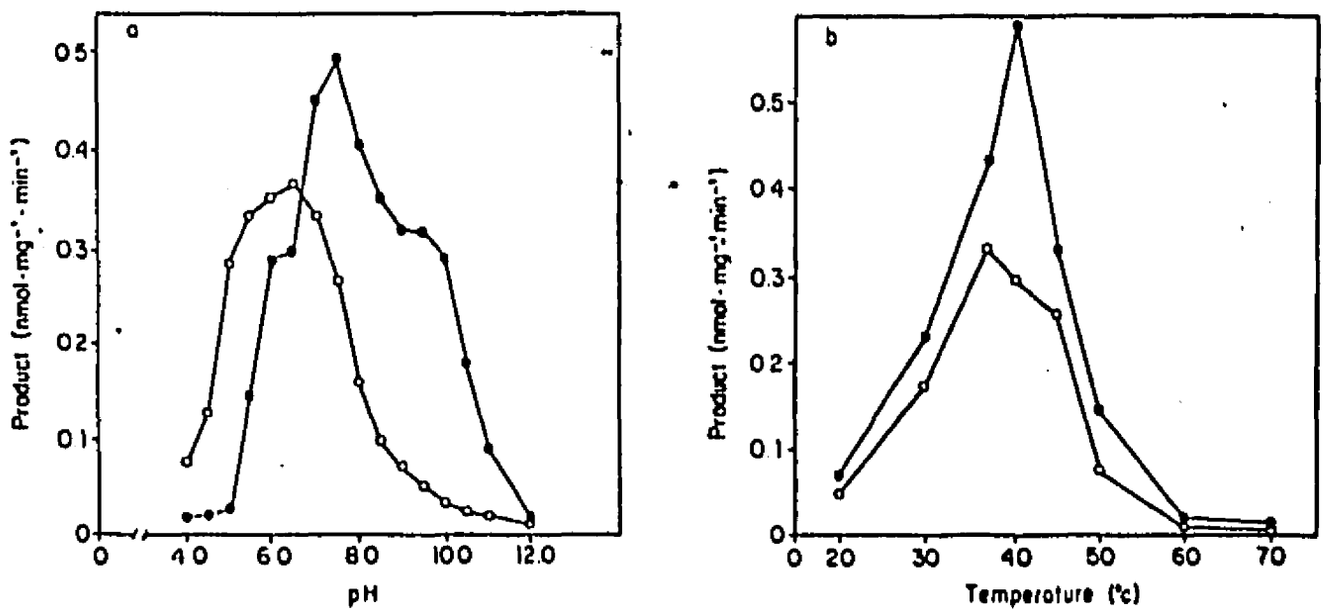

Fig. 2. *17β-hydroxysteroid dehydrogenase activity as a function of* (a) *pH and* (b) *temperature*. Incubations were performed with the pancreatic mitochondrial preparation containing 0.4 mg of protein per flask. The amount of testosterone (o—o) or androstenedione (•—•) formed was plotted.

(Fig.2b). The apparent maximal reductive activity for androstenedione was attained at 37°C, and maximal rate for testosterone oxidation was achieved at 40°C. Both, reductive and oxidative activities rapidly decreased at temperatures over 40°C until no enzymatic conversion could be measured.

Substrate Saturation Kinetics. The effect of increasing concentrations of androstenedione and testosterone (in the range of 2.0 to 80.0 μM), incubated separately, upon the initial velocity of pancreatic mitochondrial 17β-hydroxysteroid dehydrogenase is illustrated in Fig.3. The apparent Km for androstenedione was determined to be 9.5 ± 0.9 μM (SE) in phosphate buffer, pH 7.0 and 37°C.

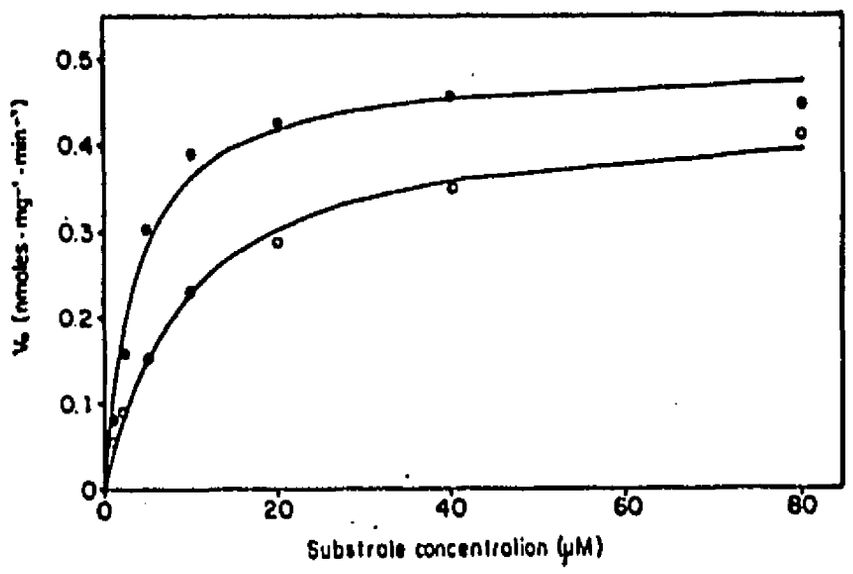

Fig. 3. *Direct plot of initial velocities as a function of steroid substrate concentration*. Androstenedione (o—o); testosterone (•—•). Protein concentration of the mitochondrial enzyme preparation was 0.4 mg ml^{-1}. Full lines are corrected curves given by the computer. Each point represents the average of at least two assays.

Using testosterone as a substrate, the apparent Km value was 3.5 ± 0.5 μM (SE) under the same conditions. Both, androstenedione and testosterone yielded an identical Vmax value of 0.4 nmol mg^{-1} min^{-1}, agreeing with the results obtained in the pH studies, in which oxidation and reduction occur at the same rate at a pH of around 7.0 (Fig.2a).

The Km values estimated in the present experiments were of the same order of magnitude as those of the 17β-hydroxysteroid dehydrogenases of porcine, rat, and human testes, which are physiologically involved in the biosynthesis of testosterone (14-16).

Although the physiological role of pancreatic 17β-hydroxysteroid dehydrogenase remains to be determined, the presence of steroid-transforming enzymes supports the hypothesis that the pancreas could be an extragonadal site of steroid hormones biosynthesis.

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