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ALFONSO LUIS HERRERA Y LA PLASMOGENIA:

Estudio de las premisas experimentales sobre una teoría autótrofa del origen de la vida.

T E S I S

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RESUMEN

Alfonso Luis Herrera (1865-1942) encaró con particular interés una de las preguntas fundamentales de la Biología: el del origen de la vida. Su preocupación por encontrar respuesta a dicho problema lo llevó durante cuarenta años a proponer y a desarrollar un campo de estudio abocado específicamente a investigar todo lo concerniente al origen del protoplasma. En su búsqueda por el origen de la célula recurrió a un sinfín de compuestos químicos intentando reproducir *in vitro* la naturaleza química y autótrofa de los primeros organismos.

El presente trabajo tiene como objetivo caracterizar los compuestos químicos sintetizados por Alfonso L. Herrera durante 1933 y descritos en sus cuadernos de trabajo como aminoácidos azufrados. Los resultados publicados muestran como a partir de una mezcla de formaldehído, tiocianato de amonio y cloruro de amonio se producen derivados químicos del ácido isopertiocíanico, el cual representa un posible intermediario en la síntesis de aminoácidos azufrados. El análisis mediante el empleo de técnicas analíticas contemporáneas confirmó parcialmente los resultados de Herrera con respecto a la presencia de aminoácidos azufrados, con un rendimiento del 2%.

INTRODUCCION

Durante el siglo XIX, el desarrollo de las diferentes ideas sobre el origen e historia de la vida caminó de la mano de los avances científicos. Con la publicación en 1859 de "El Origen de las Especies", Charles Darwin dio a conocer su teoría respecto a la evolución de los organismos por selección natural, lo que en su momento constituyó junto con factores de índole político histórico y social, el punto de transición a una concepción del mundo y de la biología nunca antes pensada. La idea que tenía Darwin sobre la aparición de las primeras formas de vida queda claramente descrita en los breves comentarios realizados en su obra el origen de las especies y registrados con mayor detalle en la correspondencia sostenida con su amigo Hooker (Fry 2000).

Comprometido con la ciencia de su época y conciente de la importancia de las discusiones sobre la aparición de las primeras formas de vida, Darwin optó por no profundizar sobre una discusión de un tema que en el momento resultaba controvertido e infructuoso. Se sabe que Darwin evitó cualquier tipo de confrontación personal con sus críticos, en especial con quienes no comulgaban con sus ideas, y más aún con aquellos que lejos de aceptar sus ideas evolutivas continuaban sosteniendo el origen de los seres vivos por generación espontánea. Sin embargo, es un hecho innegable que a partir de Darwin la perspectiva en el desarrollo de las ideas sobre el origen de la vida se mantuvieron en términos evolutivos de cambio y transformación, facilitando la apertura intelectual hacia la consolidación de programas de investigación entre los que destacan el del origen y la evolución celular, el de la biología molecular, o el de la biomedicina enfocada a los microorganismos.

La permanencia de la generación espontánea se debió en parte a su incorporación a las teorías evolutivas. En Inglaterra la abiogenesis, esto es, la formación de lo vivo a partir de lo inanimado, fue considerada una parte integral de la continuidad evolutiva descrita en la teoría de Darwin. No así en Francia, en donde la biogénesis, es decir, la formación de un ser vivo a partir de otro y representada por Louis Pasteur, constituyó un respaldo a las ideas religiosas y un triunfo del vitalismo al negar toda posibilidad a la generación espontánea.

La falta de solución al problema del origen de la vida llevó a la formulación de propuestas alternativas de explicación. Por un lado, se difundió la hipótesis metafísica de la panspermia en donde el origen y naturaleza de la vida debían su existencia a la manifestación emergente de leyes y fuerzas naturales acontecidos en otro planeta. Acorde con la eternidad de la vida la panspermia sugería que una vez que la vida apareció en el Universo ésta se diseminó en la Tierra y hacia otros mundos (Oparin 1938; Kaminga 1988; Fry 2000). El resurgimiento en el siglo XIX de la teoría de la generación espontánea y la panspermia no modificaron en nada los esquemas tradicionales de estudio por el origen de las primeras formas de vida. Sin, embargo, es un hecho innegable que este tipo de ideas nos han permitido enriquecer nuestra perspectiva de estudio por el origen de la vida y a la distancia nos permite apreciar que la falta de certeza científica mostrada por los intelectuales del siglo XIX fue en gran medida consecuencia de la inmadurez experimental para responder a un problema muy antiguo.

Fue en las postrimerías del siglo XIX, mediante las transformaciones políticas, sociales y científicas que se tuvieron nuevos criterios de investigación para el estudio del origen de la vida. Al finalizar el siglo XIX las explicaciones en Biología incorporaron metodologías de exploración limítrofes, tales como la física y la química, que a la ves generaban interrogantes como el de la esencia de la célula así como el de sus constituyentes, el protoplasma celular. Una de estas teorías emergentes fue la de la plasmogenia, instituida y generada por el célebre naturalista mexicano Alfonso Luis Herrera (Herrera 1904).

Alfonso L. Herrera pensaba que las propiedades química de los seres vivos estaban ya presentes en los primeros organismos. Demostrar como experimentalmente los coloides participaron en el protoplasma y en el origen de la vida fue el objetivo central de la plasmogenia.

La idea del origen químico del protoplasma no surge con Herrera. Concebida la teoría celular, el protoplasma parecía estar fielmente representado en la química de los coloides, y aunque en la mayoría de los círculos científicos europeos el núcleo y el citoplasma se constituyeron como parte de los proyectos de investigación biológica, en ningún caso se instituyó un proyecto de trabajo al origen de la vida.

Herrera pensaba que la naturaleza inorgánica del protoplasma en los primeros organismos (ó protobios, como los bautizó) tenía su origen en sales y sustancias coloidales inorgánicas: Herrera (1924) expresó lo siguiente:

> ....Siguiendo nuestra hipótesis hasta cierto punto fantástica podemos pensar que tales celdillas de naturaleza inorgánica, se han desarrollado procesos biológicos que han tenido como resultado el nacimiento de las materias orgánicas y la vida. Los verdaderos organismos habrían sido precedidos por seudo organismos....

El ejercicio de las prácticas químicas en las ciencias naturales llevaron a Herrera a sostener una concepción materialista y mecanicista de los fenómenos celulares. Así, en 1903 Herrera propuso la teoría de la plasmogenia, que él mismo definió como "la ciencia que estudia el origen de la forma" aludiendo con ello a la aparición el protoplasma. Herrera creía que fenómenos tales como el crecimiento celular, la nutrición, la regulación osmótica o la reproducción adquirían sentido ya no en una teoría celular, sino a la luz de las nuevas explicaciones consolidadas en las bases químicas de la vida. Así, para Herrera la naturaleza material de todo estructura sea o no viviente es, en esencia, la misma por lo que la organización y complejidad en los seres vivos o extintos no es sino la manifestación de la materia bajo la acción de leyes físicas universales.

Es esta visión pretendidamente holista con lo que la Herrera construye la plasmogenia, concebida como una teoría general de procesos biológicos y como un proyecto experimental de investigación para el estudio del origen de la vida. Sin lugar a duda el valor interpretativo que en su momento alcanzó la naturaleza coloidal del protoplasma constituyó un elemento descriptivo e integrativo para las futuras investigación sobre biología celular.

La plasmogenia considerada en un principio como una teoría cuyo objetivo era la de estudiar la composición celular se convertirá en las postrimerias del siglo XIX en una hipótesis original y alternativa a las entonces existentes sobre el origen de la vida.

Las ideas de Ernst Haeckel de la naturaleza autótrofa, amorfa y homogénea de los primeros seres vivos (Haeckel 1876) fue ampliamente conocidas y difundidas por los naturalistas europeos del siglo XIX. En Alemania Haeckel incorporó el conocimiento de las formas más simples de vida a la teoría evolutiva de Darwin, lo que influyó fuertemente en Herrera. Se dio por hecho que los proceso graduales de cambio evolutivo de un mundo inorgánico a uno orgánico eran la mejor explicación evolutiva sobre el origen de las formas más simples de vida. Las ideas de Herrera tiene también como antecedentes al célebre naturalista alemán F. W. Pflüger, quien como muchos de su tiempo pensaba que la vida era producto del calor intramolecular generado en degradación oxidativa de compuestos derivados de la albúmina viva (Pflüger 1875). Pflüger consideraba que el elemento constitutivo de la célula, la albúmina, estaba conformada principalmente por dos componentes: los inertes o estructurales, y los vivos o funcionales. La inestabilidad química de los segundos la atribuía a la presencia del grupo químico ciano (-CN), el cual, asociado a otros compuestos químicos, determinaba por su reactividad la esencia de la naturaleza viva y su formación a partir de la materia inerte.

Pflüger pensó que durante la fotosíntesis los carbohidratos tenían su origen de la reacción química de los grupos químicos –CN y CO. La reacción química entre aldehídos de Canizzaro o la de Buttlerow (1861) en la obtención de formosa parecían constatarlo.

Se tenía la idea de que la proporción de elementos químicos como el carbono, azufre, fósforo, flúor o sílice en las células estaba asociada con su abundancia en la corteza terrestre. Para Herrera, la presencia de dichos elementos y su reactividad en el protoplasma confería a los organismos una serie de propiedades vitales; entre las principales, la morfogenética (diferenciación de los diferentes tipos celulares), y la sintética como generadora de materia orgánica. Es por esta razón que las primeras descripciones sobre los procesos fotosintéticos condujeron a Pflüger y algunos de sus contemporáneos y al propio Herrera a incluir a los grupos -CN , S, y CO_2 como compuestos esenciales en la elaboración de una teoría sobre el origen autotrófico de los organismos.

En Biología el reduccionismo físico-químico practicado durante el Siglo XIX transcurrió de forma independiente a las ideas evolucionistas. La plasmogenia no fue la excepción y aunque la composición de la materia biológica y su origen estaban en discusión, la idea de Haeckel de que sólo los seres vivos y en general las células son capaces de sintetizar materia orgánica no esta experimentalmente comprobada. Con esto en mente Herrera intentó demostrar que el problema sobre la aparición de las primeras formas de vida se puede entender experimentalmente.

Como egresado en Farmacia y con un amplio conocimiento de la química de su tiempo, Herrera se inclinó en favor de un origen inorgánico de la vida, por lo que durante toda su vida intentó crearla en el laboratorio, recurriendo para ello a la repetición experimental de fenómenos controlables. Convencido de la reacción de formosa para obtener químicamente el almidón, buscó el origen autotrófico de la vida a partir del formaldehído y de compuestos químicos derivados del azufre. Así, a partir del tiocianato de amonio generó una diversidad de formas que imitaban en mucho a las identificadas en cortes histológicos de diversos organismos. En ocasiones se identificaban como células de naturaleza microbiana sin serlo, por su naturaleza azufrada este tipo de morfotipos celulares fueron bautizados como "sulfobios"

Con esta ideas en mente Herrera realizó, a petición de Giovanni Ciamician, de la Universidad de Bolonia, una serie de experimentos encaminados hacia la síntesis no biológica de compuestos orgánicos. Dichos trabajos nunca se concretaron y quedaron interrumpidos a raíz de la muerte de Ciamician. Sin embargo, al substituír el cianuro de amonio (NH_4CN) por el sulfocianuro de amonio (NH_4SCN), Herrera obtuvo no sólo aminoácidos como los entonces descritos treinta años atrás por Klages (1903), sino también aminoácidos azufrados como la cisteína o la metionina. La determinación experimental de la síntesis de aminoácidos a partir del tiocianato de amonio fue publicada por Herrera en 1933 (Perezgasga 1989). La reproducción experimental con metodologías contemporáneas de estos experimentos han sido recientemente publicados, y constituyen el tema central de esta tesis.

Los sulfobios resultaron ser el mejor modelo experimental de "La teoría sulfocianica" del origen de la vida , ya que dichos morfotipos representaban, según Herera, la formación de células en condiciones geológicamente activa y en presencia de azufre. En busca del

origen mediante la morfología celular, Herrera recurrió al empleo de diversos compuestos químicos, tales como aceite de oliva o gasolina, que una vez mezclado simulan células microscópicas. Los colpoides formaron parte de esta gama de "artefactos" celulares, y entre sus propiedades estaba no sólo el de presentar cierta similitud morfológica con las células, sino en ocasiones representar procesos biológicos celulares tales como el de motilidad, el de la osmosis o el de la división celular entre otros.

Es de llamar la atención el hecho de que el desarrollo de la teoría del protoplasma o plasmogenia, a diferencia de las propuestas de sus contemporáneos adquiere en Herrera un matiz definido y claro sobre la problemática del origen de la vida. A.L. Herrera desarrolló la teoría de la plasmogenia en México de forma independiente y ajena a todo vinculo institucional. Indiscutiblemente, como lo señaló el propio Herrera, son muchos los investigadores a los que hay que considerar como pioneros de un campo de investigación como es el de la plasmogenia. Los meritos históricos y científicos de A.L. Herrera son innegables, de una u otra manera se reconoce como miembro de una tradición de experimentadores aún vigente iniciada en la obra de plasmogenistas europeos como Otto Butschli, Stéphane Leduc, Albert y Alexandre Mary, por mencionar algunos. En Francia y España los coloidólogos se incorporan al proyecto plasmogenista bajo una crítica rigurosa y severa mostrando sus distintas ideologías sobre sus trabajos en el estudio del origen de la vida. (Catalá y Peretó 2001).

Uno de los aspecto que llama la atención en Herrera es la noción histórica y evolutiva que adoptó en sus textos con lecturas de naturalistas como Lamarck, Buffon, Haeckel y Darwin entre otros. Así la interpretación que de ellos hace lo convierte en uno de los fundadores del evolucionismo en México. Paradójicamente, ésta noción de progreso y de cambio biológico no resulta del todo evidente en los argumento metodológico implementado experimentalmente en sus ideas plasmogenistas.

CONCLUSIONES

A finales del siglo XIX las ciencias naturales afrontaron un severo dilema ante la interrogante del origen de la vida. Teorías como la panspermia, el gen y la enzima autocatalítica y heterocatalíticas (MUller 1926; Troland 1914, 1917) no respondían a la interrogante sobre el origen de la vida en la Tierra. Por otra parte, las ideas evolutivas de Lamarck o Darwin sostenían la transición gradual de un mundo inorgánico a uno orgánico. Haeckel sostuvo que las primeras formas vivientes tuvieron su origen en el plasma celular. El mexicano Alfonso Luis Herrrera encaró experimentalmente las sugerencias de Haeckel con una teoría novedosa, "La plasmogenia". En ella Herrera intentó recrear *in vitro* la naturaleza química de la vida.

Las ideas de Pflüger sobre la importancia del ácido cianhídrico en la catálisis biológica fueron la punta de lanza para que Herrera desarrolla hacia 1920 "La teoría sulfociánica" del origen de la vida (Herrera 1942). Dicha teoría sugiere que la estructura físico-química del protoplasma celular tiene su origen en los compuestos químicos derivados del azufre y el ácido cianhídrico. Así, para Herrera la reacción entre compuestos químicos azufrados además de generar imitaciones de células producía compuestos orgánicos tales como azúcares, aminoácidos, pigmentos orgánicos.

En 1933 Herrera describió en sus cuadernos de trabajo, como a partir del tiocianato de amonio se obtienen los aminoácidos alanina, cisteina y metionina y recientemente reproducidos con metodologías contemporáneas (Perezgasga et. al 2003; Silva, et al, en prensa).

Herrera pensaba que una vez generada la matriz celular la fijación natural del bióxido de carbono y la reducción del formaldehído se realizaría como en los organismos autotróficos de hoy día.

Herrera intentó la formación de un protoplasma autotrófico mediante la utilización de precursores químicos, que desde su punto de vista generaba el mejor modelo representativos de un sistema mínimo de vida. Para ello, confeccionó químicamente la formación microscópica de estructuras equiparables en crecimiento, movilidad o difusión osmótica a células. Las clasificó en dos grupos: (a) colpoides, los cuales eran producidos

mediante aceite de olivo, gasolina entre otros, y (b) sulfobios, resultado de la mezcla del tiocianato de amonio (NH_4SCN) y el formaldehído (H_2CO).

A su muerte, acontecida el I7 de septiembre de I942, Herrera contaba en su haber con más de diez mil experimentos y ensayos realizados durante cuarenta años de trabajo, la mayoría de ellos elaborados con sustancias coloidales.

La síntesis química de compuestos biológicos simulando condiciones de la Tierra primitiva constituye una línea de investigación central de la evolución química que junto con la existencia de compuestos orgánicos identificados en cuerpos estelares de 4,000 millones de años (nubes de gas y polvo interestelares, cometas y meteoritos) representan una evidencia adicional de los fenómenos físicos y químicos previos al origen de la vida..

Los aminoácidos son uno de los compuestos más abundantes de los productos químicos sintetizados abióticamente, sin embargo y a pesar de su importancia aún hoy día no se cuenta con una síntesis abiótica satisfactoria de aminoácidos azufrados. Es en este sentido las investigaciones realizadas por A. L. Herrera son discutidas y comentadas en los artículos aquí editados.

La mezcla de reactivos utilizada por A. L. Herrera incluye al tiocianato de amonio, el cual hoy día se sabe está presente en chimeneas hidrotermales y medio interestelar (Mukhin, 1974; Dowler y Ingmanson 1979). El formaldehído también incluido en la mezcla de reacción es también abundante en cometas y partículas del medio interestelar (Irvine 1998).

En presencia del cloruro de amonio la reactividad del tiocianato de amonio con el formaldehído genera una gran variedad de productos. Es a partir de estos compuestos que se obtuvieron derivados de aminoácidos azufrados. La síntesis prebiótica para aminoácidos azufrados con un rendimiento de 2% se realizó mediante la irradiación por descargas eléctricas de una mezcla de ácido sulfhídrico en combinación con nitrógeno y carbón. (Bhadra y Ponnamperuma 1986). Por otra parte, Steinman et. al (1968) irradiando una solución de tiocianato de amonio a 0.1 M reportaron rendimientos del 1%. Con un rendimiento del 2% en el presente trabajo se obtuvo la síntesis de cinco aminoácidos dos de ellos correspondientes a aminoácidos azufrados. El mecanismo de síntesis de aminoácidos es complejo, y su obtención probablemente fue resultado de la formación del HCN a través de la descomposición hidrolítica del tiocianato de amonio. Como evidencia de la reacción

de síntesis prebiótica se obtuvieron aminoácidos no proteínicos como el ácido ß-aminoisobutírico (ß-AIB), con lo que se excluye su procedencia como contaminante biológico. Algunos de los compuestos no identificados en HPLC probablemente corresponden a aminoácidos no proteínicos como por ejemplo la tioglicina. Los resultados aquí expuestos confirman la síntesis de aminoácidos azufrados reportados por Herrera. Es importante señalar que sus experimentos se realizaron bajo la óptica de un origen inorgánico y autotrófico de la vida. El bajo rendimiento del 2% aquí señalado deberá ser reevaluado para poder explicar su origen y permanencia una vez que se realicen análisis sobre cinética y estabilidad de síntesis y degradación de compuestos simulando condiciones de Tierra primitiva.

Las nuevas tecnologías metodológicas confirmaran a futuro los trabajos experimentales publicados en el siglo pasado por Herrera. Los resultados de los experimentos aquí descritos de Herrera fortalecen la construcción histórica de la ciencia mexicana del siglo XIX y nos permite entender las razones del abandono total por una práctica científica experimental. Para Herrera la plasmogenia significó en su momento el reconocimiento internacional a su ardua labor experimental, por ello es que la contribución de sus ideas lo consolidan en la actualidad como uno de los fundadores de la evolución química en el campo del origen de la vida. Entre sus logros individuales está la fundación de la Sociedad Internacional de la Plasmogenia. El esfuerzo que le significó dicho trabajo se vio retribuido con el establecimiento de la Gaceta de la Plasmogenia y el Bulletin du Laboratoire et de la Societe Internacionale de Plasmogenie. El valor historeográfico que representa el rescate de estos experimentos contribuirá, entre otras cosas, a la consolidación sobre posibles rutas de síntesis química prebiótica.

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Apéndice:

Becerra A, Silva E, Lloret L, Islas S, Velasco A. and Lazcano A. (2000) Molecular biology and the reconstruction of microbial phylogenies: Des liaisons Dangereuses? *In: J. Chela Flores et. al. (eds)* Astrobiology, 135-150. Kluwer Academic Publishers. Netherlands.

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A REEXAMINATION OF ALFONSO HERRERA'S SULFOCYANIC THEORY ON THE ORIGIN OF LIFE

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1.Introduction

Based on Pflüger's proposal on the role of CN-containing derivatives in biological catalysis (Pflüger, 1875), 2Alfonso L. Herrera developed in the late 1920's the sulfocyanic theory of the origin of life (Herrera 1942). According to this idea, the physical structure of cellular plasma was derived from sulfur- and CN- containing compounds that formed a molecular matrix within which the primordial fixation of CO_2 took place via its reduction to H_2CO.

As described in his extensive bibliography (Beltrán, 1968), Herrera achieved the formation of microscopic structures which he claimed were comparable to cells, due to their growth, motility, and osmotic properties. He promptly divided then into two major groups: (a) *colpoids*, which were produced when olive oil, gasoline, and other complex molecules were used; and (b) *sulphobes*, which resulted from the mixture of NH_4SCN and H_2CO (Herrera, 1942). After many trials, Herrera found that the best starting material for the formation of his sulphobes was ammonium thiocyanate, which he dissolved in formaline and spread in thin layers until evaporation (Herrerra 1942). According to Herrera, the reactions of these precursors gave rise not only to several kinds of cell-like microstructures, but also to (a) starch; (b) two uncharacterized amino acids; (c) globules of red, green and yellow pigments; and (d) what he described as a "proteinoid condensation product".

As shown by photocopies of some of his laboratory notes (available upon request), by 1933 Herrera was convinced that he had achieved the synthesis of glycine, cysteine and cystine. The formation of these compounds, which Herrera synthesized using formaldehyde and ammonium thiocyanate as starting materials, were based on the glycine synthesis from formaldehyde and potassium cyanide reported by Klages (1903) and Ling and Nanji (1922). Although Herrera (1942) mentioned the synthesis of "starch, and at least a two amino acids", he did not list them nor characterize the other products he obtained. Here we attempt to do so, based on the repetition of some of his experiments and the use of modern analytical tools.

2. Materials and Methods

We first mixed 20 ml of 37% formaldehyde (0.05 M final) with 36 g of ammonium chloride (0.067 M final) and put this solution into a three-mouth flask that was kept on ice. The mixture was stirred all the time with a mechanical stirrer. We then prepared a 0.055 M ammonium thiocyanate solution that was poured for 30 min with a separator funnel. When half of the thiocyanate solution was added, 25 ml of glacial acetic acid was dropped and the solution was stirred for two more hours. The temperature was always kept below 10^0 C. A precipitate was formed with yellow and white crystals. The mixture was filtered and the crystals were air-dried. By a fractionated precipitation (see Table I), we obtained two more precipitates (the second one contained yellow crystals, and the third one yellow and white crystals that were separated). Based on their infrared spectra, we decided to work with the precipitate 1 and precipitate 3 white crystals. These were analyzed by infrared spectroscopy, a size-exclusion separation method, and HPLC. HPLC chromatography was done at the Amino Acids Unit of the Instituto de Investigaciones Biomédicas, UNAM, using a Beckman Instruments HPLC series chromatograph. The column used was a C_{18} with a particle size of 5 μ. The analyses were done at room temperature at a wavelength of 340 nm. The retention time of various amino acids was determined using a Beckman reference mixture. The fractions of precipitates 1 and 3 were hydrolyzed with sequential grade HCl (Pierce), acetic acid for HPLC and water HPLC grade, derivatized with ortho-phthaldialdehyde (Lindroth and Moppen, 1979) and analyzed for the presence of amino acids (Ladrón de Guevara *et al.*, 1985). In order to identify the amino acids that could be present in the reaction mixture, the white crystals of precipitates 1 (in two runs of 500 mg each) and 90 mg of precipitate 3 were first purified by a size exclusion separation method using a SP-sephadex 25 column.

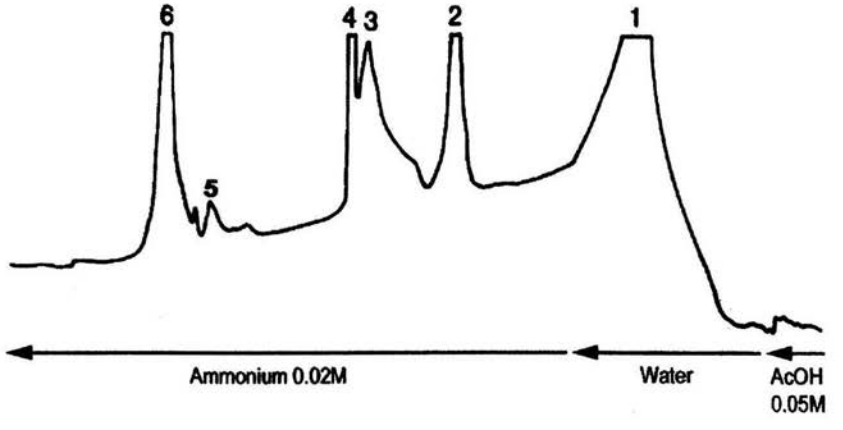

Fig.1 Size exclusion separation of products from precipitate 1, using a SP-Sephadex cm-25 column. Peak 1 corresponds to the thiocyanate ion that did not react. Peaks 2 to 6 were analyzed separately for amino acid content. HPLC identification of 1. glycine; 2. alanine; 3. cysteine; 4. methionine. Amino acids were identified by their retention times as described in he text.

3. Results

The HPLC chromatograms shown in Figure 1 indicate the presence of several compounds whose retention times correspond to glycine, alanine, cysteine, and methionine. Of these, only cysteine and methionine are sulfur containing amino acids.

Although we analyzed standard samples of other sulfur-containing amino acids, such as taurine and cystine, the peaks obtained in the chromatograms do not correspond to their retention times. Other unidentified peaks in the chromatogram could correspond to non-proteinic amino acids. The amino acid formation yield was low and was determined by the electronic integration of the peak area Perezgasga et al 2004). The same amino acids were observed in both the hydrolyzed and non-hydrolyzed fractions, although the yield was higher in the former one.

Table I. Physical properties of the precipitates formed in the reaction mixture.

Pp	Weight	Colour	Melting point (°C)	Comments
1	24.92g	Yellow	160-185	Not pure, with yellow and white cristals.
2	4.05g	Yellow	155-190	
3	0.32g	White	190-260	
3	0.30g	Yellow	115-135	

4. Discussion and conclusions

Like many of his contemporaries, Herrera was convinced that the first living beings had been autotrophes. The popularity of Pflüger's (1875) ideas on biological catalysis had led Herrera to believe that CN derivatives played an essential role in biochemical processes and, hence, that cyanogen and its derivatives must have been present at the origin of life. Led by the schemes which suggested at the time that H_2CO was a central intermediate in the photosynthetic fixation of CO_2, Herrera attempted the laboratory formation of an autotrophic protoplasm by mixing the precursors he thought were essential for a minimal living system. Thus, it was not because of foresight that he employed compounds which are nowadays recognized as potential components of the prebiotic environment. Instead, he should be recognized as a careful worker, whose deep knowledge of the major theories of his contemporaries led him to study the origins of life within the framework of his times.

Because of the assumption of primordial photosynthesis, formaldehyde was one of the main components in Herrera's sulfocyanic theory. It is generally accepted that H_2CO was present in the primitive Earth (Wills and Bada, 2000), and ammonium thiocyanate is produced in hydrothermal vents (Mukhin, 1974; Dowler and Ingmanson, 1979). Since Herrera was following the reactions described by Klages (1903) and Ling and Nanji (1922), he was actually performing a variation of a Strecker synthesis, in which ammonium thiocyanate could take the place of NH_4CN, and where the hydrolysis of the nitrile was achieved by boiling with a highly concentrated solution of barium hydroxide (Ling and Nanji, 1922). Our results here suggest that in the experiments which we have performed following the procedures described by Herrera, four amino acids were synthesized: glycine, alanine, cysteine and methionine, with a total yield of 2%. Alanine was the most abundant amino acid in all samples analyzed.

The preliminary results presented here suggest that a variation of the Strecker synthesis involving formaldehyde and ammonium thiocyanate could lead to low yields of amino acids, including sulfur-containing amino acids. Alternatively, it is possible that the amino acids reported here, including cysteine and methionine, are the outcome of an hydrolysis of oligomers that could be formed by the self-condensation of SCN, by a mechanism equivalent to that described by Ferris *et al* (1978). These possibilities should be re-evaluated once mass-spectrometry identifications and kinetic studies on the stability of synthesis and degradation of these compounds under the conditions described here are performed

Although the starting materials used by Herrera were determined by his autotrophic hypothesis on the origin of cells, our results show that his experiments may provide insights on the abiotic synthesis of sulfur-containing amino acids within the framework of a heterotrophic emergence of life.

Acknowledgements

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The Editors

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Membranes and prebiotic evolution: compartments, spatial isolation and the origin of life

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I. Introduction

Was compartmentalization essential for the appearance of life? While it can be argued that encapsulation within lipidic compartments of replicative and catalytic molecules of prebiotic origin would have favoured individuality and selection, it must be underlined that how the transition from the non-living to the living took place is still far from being understood. In addition, it must be underlined that the attributes of the first living organisms are unknown. They were probably simpler than any cell now alive, and may have lacked not only protein-based catalysis, but perhaps even the familiar genetic macromolecules, with their ribose-phosphate backbones. It is possible that the only property they shared with extant organisms was the structural complementarity between monomeric subunits of replicative informational polymers, e.g. the joining together of residues in a growing chain whose sequence is directed by preformed polymers. Such ancestral polymers may have not even involved nucleotides. Accordingly, the most basic questions pertaining to the origin of life relate to much simpler replicating entities predating by a long series of evolutionary events the oldest recognizable heat-loving prokaryotes represented in molecular phylogenies.

As discussed elsewhere (Lazcano, 2001), the lack of an all-embracing, generally agreed definition of life sometimes gives the impression that what is meant by its origin is defined in somewhat imprecise terms, and that several entirely different questions are often confused. For instance, until a few years ago the origin of the genetic code and of protein synthesis were considered synonymous with the appearance of life itself. This is no longer a dominant point of view; the discovery and development of the catalytic

activity of RNA molecules has given considerable support to the idea of the 'RNA world' --a hypothetical stage before the development of proteins and DNA genomes during which alternative life forms based on ribozymes existed (Gesteland *et al.* 1999). This has led many to argue that the starting point for the history of life on Earth was the *de novo* emergence of the RNA world from a nucleotide-rich prebiotic soup. Others with a more skeptical view believe that it lies in the origin of cryptic and largely unknown pre-RNA worlds. There is even a third group that favours the possibility that life began with the appearance of chemoautotrophic autocatalytic metabolic networks, lacking genetic material.

Despite the seemingly insurmountable obstacles surrounding the understanding of the origin of life (or perhaps because of them), there has been no shortage of discussion about how it took place. Not surprisingly, several alternative and even opposing suggestions have been made regarding how life emerged and whether membranes were essential or not for the appareance of living beings. As discussed here, although the classical version of the hypothesis of chemical evolution and primordial heterotrophy (Oparin 1924, 1938) needs to be updated, it still provides the most useful framework for addressing the issue of emergence of life. Alternative theories, such as the autotrophic theory proposed by Wächtershäuser (1988) have been discussed elsewhere (Lazcano, 2001; Bada and Lazcano, 2002), and will not be considered here. The basic tenet of the heterotrophic theory is that the maintenance and reproduction of the first living systems depended primarily on prebiotically synthesized organic molecules, and as discussed below, lipidic molecules were very likely part of this inventory of compounds. However, while the role of membranes in the origin of life has been considered essential in some

proposals like that of Oparin (1924, 1938), others who have followed the assumption of prebiotic synthesis and accumulation of organic compounds as a prerequisite for the origin of life, have different viewpoints.

II. Prebiotic synthesis and the heterotrophic origin of life

It is unlikely that data on how life originated will be provided by the palaeontological record. There is no geological evidence of the environmental conditions on the Earth at the time of the origin of life, nor any fossil register of the evolutionary processes that preceded the appearance of the first cells. Direct information is lacking not only on the composition of the terrestrial atmosphere during the period of the origin of life, but also on the temperature, ocean pH values, and other general and local environmental conditions which may or may not have been important for the emergence of living systems.

The idea of life as an emergent feature of Nature was widespread during the last century, but it was not until Oparin (1938) proposed that first living systems were heterotrophic microorganisms that resulted from the evolution of abiotically synthesized organic compounds and the formation of a self-sustaining supramolecular systems, that the study of the origin of life was transformed from a purely speculative discussion into a workable research program. Today scientific efforts in this field are not necessarily oriented towards the *in vitro* production of a living system, but rather towards the construction a coherent historical narrative by weaving together a large number of miscellaneous observational findings and experimental results.

The hypothesis of chemical evolution is supported not only by a number of laboratory simulations, but also by a wide range of astronomical observations and the analysis of samples of extraterrestrial material. These include the existence of organic molecules of potential prebiotic significance in interstellar clouds and cometary nuclei, and of small molecules of considerable biochemical importance that are present in carbonaceous chondrites. The copious array of amino acids, carboxylic acids, purines, pyrimidines, hydrocarbons, and other molecules which have been found in the 4.5 x 10^9 years-old Murchison meteorite and other carbonaceous chondrites gives considerable credibility to the idea that comparable syntheses took place in the primitive Earth (Oró et al., 1990; Ehrenfreund, et al. 2002).

There is also strong experimental support for the idea of prebiotic formation of organic molecules. The first successful synthesis of biochemical compounds under plausible primordial conditions was accomplished by the action of electric discharges acting for a week over a mixture of CH_4, NH_3, H_20, and H_2, yielding a racemic mixture which included several proteinic and non-proteinic amino acids, as well as hydroxy acids, urea, and other organic molecules (Miller, 1953). A few years later, Oró (1960) showed that adenine, a purinic compound that plays a central role in both genetic processes and biological energy utilization, was a major product of the non-enzymatic condensation of HCN, which in turn is an abundant constituent of interstellar clouds and cometary nuclei. The potential role of HCN as a precursor in prebiotic chemistry has been further supported by experimental evidence showing that the hydrolytic products of its polymers include amino acids, purines, and orotic acid, an intermediate in the biosyntheses of uracil and cytosine, two major

components of RNA, indicating that diverse biochemical compounds could had been formed simultaneously from simple reactants (Ferris et al., 1978). Laboratory syntheses under possible primitive conditions of other organic compounds of biochemical significance, such as tricarboxylic acids, alcohols, and a number of coenzymes has been reviewed elsewhere (Oró et al., 1990; Miller and Lazcano, 2002).

There can be little doubt that amphiphilic molecules were also present in the prebiotic environment. Lipids are polar derivatives of hydrocarbons, and the abiotic synthesis of the later has been known since the late 19th century. Unfortunately, long-chain linear hydrocarbons and fatty acids are relatively difficult to synthesize under simulated prebiotic conditions (Deamer et al., 1994; Maynard Smith and Szathmary, 1995; Norris and Raine, 1998). Although high yields of several lipidic molecules, including phosphatidic acids, phosphatidylethanolamine, and phosphatidylcholine have been reported in abiotic synthesis starting from simple precursors and following a sequence of step-by-step reactions (Oro et al., 1978), an abiotic source of inorganic phosphate on the primitive Earth in not inmediatly apparent. However, certain components of carbonaceous meteorites can self-assemble into membranous structures, suggesting a prebiotic accumulation of lipidic compounds due to the infall of extraterrestrial material of meteoritic origin (Deamer et al., 1994).

The above results suggest that the prebiotic soup must have been a bewildering organic chemical wonderland, but it could not include all the compounds or the molecular structures found today even in the most ancient extant forms of life --nor did the first cells spring completely assembled, like Frankestein's monster, from simple precursors present in

the primitive oceans. The fact that a number of chemical constituents of contemporary forms of life can be synthesized non-enzymatically under laboratory conditions does not necessarily imply by itself that they were also essential for the origin of life, or that they were available in the primitive environment. Moreover, the lack of agreement on the chemical constituents of the primitive atmosphere has also led to major debates. Although it is generally accepted that free oxygen was absent, many planetologists favor the possibility that it consisted of much less-reduced gases such as CO_2, N_2, and H_2O (Kastings, 1993), while prebiotic chemists prefer more reducing mixtures (Lazcano and Miller, 1996).

The correlation between the compounds which are produced in prebiotic simulations and those found in carbonaceous meteorites (Becker te al., 2002) is too striking to be fortuitous, and strongly supports the contention that such molecules were part of the chemical environment from which life evolved. However, the leap from biochemical monomers and small oligomers to membrane-bounded cells is enormous. There is a major gap between the current descriptions of the primitive soup and the appearance of non-enzymatic replication. Solving this issue is essential to our understanding of the origin of the biosphere: regardless of the chemical complexity of the prebiotic environment, life could not have evolved in the absence of a genetic replicating mechanism insuring the maintenance, stability, and diversification of its basic components.

III. The search for the RNA world

How the ubiquitous nucleic acid-based genetic system of extant life originated is one of the major unsolved problems in contemporary biology.

The discovery of catalytically active RNA molecules gave considerable credibility to prior suggestions of that the first living organisms were largely based on ribozymes, an hypothetical stage called the RNA world (Gilbert, 1986; Joyce, 2002). This possibility is now widely accepted, but the chemical lability of RNA components suggests that this molecule was not a direct outcome of prebiotic evolution (Orgel, 2003). Moreover, many different (and sometimes even opposing) versions of the RNA world coexist, although its original formulation involved encapsulation of catalytic and replicative RNA molecules within liposomes (Alberts, 1986; Gilbert, 1986; Lazcano, 1986).

It is unlikely that wriggling RNA molecules were floating in the primitive ocean, ready to be used as primordial genes. As reviewed elsewhere (Lazcano and Miller, 1996), from the chemical viewpoint the RNA world faces major problems, which include the origin of its ribose moiety, the rapid decomposition of this and other sugars under primitive conditions, and the availability of polyphosphates and phosphate esters, which are not prebiotic reagents. These difficulties have led to proposals of pre-RNA worlds, in which informational macromolecules with backbones different from those of extant nucleic acids may have also been endowed with catalytic activity, i.e., with phenotype and genotype also residing in the same molecules. The nature of the genetic polymers and the catalytic agents that may have preceded RNA is of course unknown (Orgel, 2003). One candidate are the so-called peptide nucleic acids, or PNAs, which are linear polymers in which the sugar-phosphate backbones of RNA and DNA are replaced by uncharged peptide-like backbones formed by achiral amino acid units linked by amide bond, to which the bases are covalently attached (Nielsen, 1993). However attractive PNAs or other candidates may be, the

origin of non-enzymatic replication remains a major, unsolved problem. Nonetheless, experimental models provide interesting insights. Enhancement of monomer concentration in experimental systems simulating a drying lagoon has achieved a successful surface-bound template polymerization up to 53 nucleotides (Ferris et al., 1996), and the chiroselective self-assembly of long homochiral oligomers of nucleic acid analogues from racemic mixtures of smaller chains into oligomers has been reported (Bolli et al., 1997).

There is evidence suggesting that replication may be a widespread phenomenon that includes chemical systems lacking the familiar nucleic acid structure (Orgel, 1992). This possibility is supported by (a) a 32-residue alpha-helical peptide which can template and catalyze its own synthesis from activated smaller fragments under aqueous conditions (Lee et al., 1996); (b) a horseshoe-shaped product of the chemical reaction between an amino-adenosine and a complex aromatic ester, whose product enhances the formation of similar molecules in a non-aqueous solvent (Hong et al., 1992); and (c) synthetic micelles containing lithium hydroxyde and stabilized by an octanoid acid derivative, which swim in an organic solvent that acts as a substrate for the formation of additional micelles (Bachmann et al., 1992). While it is unlikely that these non-informational autocatalytic systems are ancestral to our own DNA-based cellular reproduction, their diversity suggests that chemical replicative systems may be much more widespread in Nature than previously thought.

IV. Membranes and precellular evolution

It is frequently argued that a decisive step towards the emergence of the first living systems was the appearance of membrane-enclosed polymolecular systems, since semipermeable prebiotic membranes would have favoured (1) the cooperative interaction between different catalytic and replicative molecules, avoiding their dispersal, and opening the possibility of specific-surface chemistry processes; (2) the creation of internal microenvironments substantially different from the exterior milieu maintained by (at least partailly) selective transmembrane transport; and (3) the preferential accumulation and, eventually, differential multiplication of self-sustaining replicating systems.

Although many different models of precullar systems have been suggested the most significant may be in fact liposomes. Lipidic membranes are an essential component of cells, and amphiphilic molecules have been formed abiotically (Hargreaves and Deamer, 1978a,b; Epps et al., 1978; Rao et al, 1987). Moreover, the presence of lipids in the primitive environment is further supported by the existence of membrane-forming non-polar molecules detected in samples of the Murchison meteorite (Deamer, 1985; 1998; Deamer and Pashley, 1989).

Bilayered liposomic structures can easily autoassemble from a wide variety of lipidic molecules under both physiological and putative prebiotic conditions (Deamer and Barchfeld; Cullis and Hope, 1985; Walde et al., 1994; Chakrabarti et al., 1994). Prebiotic liposomes could have formed from small, single-chain, ionic, linear fatty acids (Hargreaves and Deamer, 1978a,b). Encapsulation of replicative and catalytic molecules could have been driven by periodic environmental changes such as dehydration-hydration cycles (Deamer and Barchfeld, 1982), enhanced by metallic

cations (Baeza et al., 1987), basic polypeptides (Jay and Gilbert, 1987), and polyamines derived from the nonenzymatic decarboxylation of basic amino acids. Neither the size or the functional properties of RNA molecules or polyribonucleotides appear to be altered by the encapsulation process, which can take place in the presence of histidine and prebiotic condensing agents such as cyanamide (Oro and Lazcano, 1990).

It is reasonable to assume that once prebiotic liposomes were formed by the self-assmebly of simple amphiphilic lipids in the presensce of a large variety of biochemical monomers and oligomers, new physicochemical properties could result from the interactions between the componentes of these polymolecular systems. This is not purely speculative; that interactions between liposomes and different water-soluble polypeptides lead to major changes in the morphology and permeability of liposomes of phosphatidyl-L-serine, and to a transition of poly-L-lysine from a random coil into an α-helix that exhibits hydrophobic bondign with the lipidic phase has been documented in the laboratory (Hammes and Schullery, 1970). Comparable interactions may have taken place between lipids and different oligomers of prebiotic origin, leading to changes in the stability and catalytic properties of precellular systems. This hypothesis is consistent with the suggestion that an energy source for nutrient transport and chemical activation processes may have been provided by chemiosmotic proton gradients formed by simple pigments asymmetrically oriented in the lipidic layer (Deamer and Oro, 1980; Morowitz et al., 1988).

The formation of liposomes raises the question of the uptake of small water-soluble molecules that cannot penetrate lipidic membranes (Lazcano et al., 1992; Wächtershäuser, 1992). It can be argued that nonselective

pores may have existed in primitive membranes, since it is known that these structures are formed in mixed lipid bilayers (Robertson, 1983), when poly-L-serine is added to liposomes (Hammes and Schulley, 1970), and when phosphatidate is present in the lipid mixture (Baeza et al., 1990). Complex transporters were of course absent during the prebiological stages of evolution, but carrier-mediated diffusion may have taken place. It has been suggested that a rudimentary transport mechanism may have existed, involving facilitated diffusion of complexes between aldehydes, amines, and metal ions with amino acids, sugars, and nucleotides, repectively (Stillwell, 1976, 1980; Stillwell and Rau, 1981). This possibility have gained support from experiments that have shown that neutral forms of amino acids go accross lipidic membranes much more easily than their charged form (Chakrabarti and Deamer, 1994).

V. Some biological problems

It is not known how proton gradients originated and became coupled with iones and directionality. However, transporters must have appeared early in biological evolution. In order to study ion conduction across biological membranes, Lear et al (1988) synthesized model oligopeptides that were large enough to span the hydrocarbon phase of the lipid bilayer. Quite significantly, they found that two small amphiphilic peptides with a simple repetitive structure behaved as more complex biological ion channels do. A 21-residue peptide with the sequence H_2N-(Leu-Ser-Ser-Leu-Leu-Ser-Leu)$_3$-$CONH_2$ formed ion channels whose permeability and lifetime resembled that of the acetylcholine receptor, while an equally small peptide with the sequence H_2N-(Leu-Ser-Leu-Leu-Leu-Ser-Leu)$_3$-$CONH_2$ produced proton-selective channels. These experiments were not performed within

an evolutionary context or under prebiotic conditions (Lear et al., 1988). However, they illustrate how small simple oligopeptides or prebiotic orgin, or synthesized by primitive cells with limited coding abilities, could have been involved in selective ion transport across primitive membranes.

The origin of transport mechanisms is related to the appearance of transduction systems and energy-producing mechanisms, i.e, to bioenergetic processes which lie at the very basis of metabolism (Holden, 1968; Maloney and Wilson, 1985). It is generally assumed that membranes provided the necessary separation between the internal microenvironment and the external surroundings which were to maintain higher reaction rates inside precellular systems. This has been clearly demonstrated by the enzyme-mediated synthesis of poly(A) within the boundaries of coacervate drops (Oparin, 1971) and, more recently, in phospholipid or oleicacid/oleate vesicles (Walde et al., 1994; Chakrabarti et al., 1994). However, it is not known how primordial energy transduction systems became coupled with polymerization reactions involving the components of primordial genetic polymers.

The prebiotic synthesis of lipids implies that early membranes were formed, in a very literal sense, from the fat of the land (or of the oceans). Hence, reproduction of the first cells would have been hindered by the exhaustion of this supply of lipidic material of prebiotic origin. A possible primitive synthesis of fatty acids from glycoaldehyde has been suggested (Weber, 1991), but very little is known of the evolutionary steps that led to the development of biosynthetic pathways of lipids. Since these molecules are a prerequisite to the origin and maintenance of cells, and since acetyl-coA is mandatory in contemporary biosynthesis of fatty acids (Vance and

Vance, 1985), one possibility is that the original activation of acetate may have resulted from its interaction with catalytic RNA molecules (Lazcano, 1986).

VI. Final remarks

There has been no shortage of discussion about how the prebiotic soup formed and the transition to the origin of life took place. However, it is likely that no single mechanism can account for the wide range of organic compounds that may have accumulated on the primitive Earth, and that the prebiotic soup was formed by contributions from endogenous syntheses in a reducing atmosphere, metal sulphide-mediated synthesis in deep-sea vents, and exogenous sources such as comets, meteorites and interplanetary dust (Miller and Lazcano, 2002). As summarized here, the existence of different abiotic mechanisms by which biochemical monomers can be synthesized under plausible prebiotic conditions is well-established. The wide range of experimental conditions under which organic compounds can be synthesized demonstrates that prebiotic syntheses of the building blocks of life are robust, i.e., the abiotic reactions leading to them do not take place under a narrow range defined by highly selective reaction conditions, but rather under a wide variety of experimental settings. The robustness of this type of chemistry is supported by the occurrence of most of these biochemical compounds in the Murchison meteorite (Miller and Lazcano, 2002), including membrane-forming lipidic molecules (Deamer et al., 1994).

It is very attractive to assume that compartamentalization within liposomes formed by amphiphilic molecules of prebiotic origin was essential for the

emergence of life. However, other alternatives include sequestering of catalytic and replicative molecules within prebiotic compartments made of hydrobic amino acid polymers (Lehmann and Kuhn, 1984), simple terpenoids (Ourisson and Nakatani, 1994), or alternating β sheet-forming polypeptides (Brack and Orgel, 1975). As noted by Joyce (2002), alternative membrane-free systems such as the association of RNA (or its pre-RNA precursors) and other molecules to surfaces via transient covalent or non-covalent interactions (Gibson and Lamond, 1990) and passive compartmentalization within aerosol drops (Dobson et al., 2000) or in rock pores can also be envisoned. However, even if spatial isolation of the first replicative systems did not involve lipidic membranes, the transition from hypothetical precellular systems into the extant biological membranes and the biosynthesis of their lipidic componentes must have taken place very early in evolution. Testable descriptions of how such transition took place require coherent proposals involving ribozyme-mediated reactions or semi-enzymatic synthesis involving less-specific biological catalysts. Understanding how this took place remains a major, unsolved problem in our understanding of the emergence of life.

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The sulfocyanic theory on the origin of life: towards a critical reappraisal of an autotrophic theory

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Abstract: In the early 1930s, Alfonso L. Herrera proposed his so-called sulfocyanic theory on the origin of life, an autotrophic proposal on the first living beings according to which NH_4SCN and H_2CO acted as raw materials for the synthesis of bio-organic compounds inside primordial photosynthetic protoplasmic structures. Although the work of Herrera is frequently cited in historical analysis of the development of the origin of life studies, very little attention has been given to the chemical significance of the reactions he published. In this paper we report the results of our search for amino acids obtained from a reactive mixture used by Herrera from 1933 onwards. Chromatograms using the high-pressure liquid chromatography (HPLC) technique suggest the presence of several amino acids, the total yield being 2% of the initial thiocyanate used. Preliminary identification based on HPLC retention times suggests the presence of glycine, alanine, cysteine and methionine. Alanine was the most abundant amino acid in all samples of fractionated material analysed. Although the starting materials used by Herrera were determined by his autotrophic hypothesis on the origin of cells, our results show that his experiments may provide insights into the abiotic synthesis of sulfur-containing amino acids within the framework of a heterotrophic emergence of life.

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Key words: amino acid syntheses, ammonium thiocyanate, formaldehyde, Strecker synthesis, sulfocyanic theory.

Introduction

Based on the work of Traube (1867), Bütschli (1894), Leduc (1910), Loeb (1912) and others, during the early 20th century a number of scientists attempted to understand the nature of cellular life as the outcome of physico-chemical forces ruling a primordial photosynthetic protoplasm, a system which was believed by many to represent the starting point of biological evolution (Fox Keller 2002). Foremost among these was the Mexican naturalist Alfonso L. Herrera (1868–1942), who during his lengthy scientific life attempted the laboratory synthesis of primitive photosynthetic cells. Several stages can be distinguished in Herrera's scientific career, including his attempts to produce lifelike structures from various combinations of substances and inorganic fluids (Cartwright *et al.* 2002; Garcia-Ruiz *et al.* 2002; Lazcano 2003), but certainly one of the most interesting corresponds to the development, based on the influence of Pflüger's proposal on the role of aldehydes and CN-containing derivatives in biological catalysis (Pflüger 1875), of what he 'the sulfocyanic theory of the origin of life'. According to this hypothesis, the physical structure of cellular plasma was derived from sulfur-containing compounds that formed part of a molecular matrix within which the primordial fixation of CO_2 took place via its reduction to H_2CO.

During his lifetime Herrera combined numerous compounds in an attempt to understand the chemical origins of protoplasm. As reported in his extensive bibliography (Beltrán 1968), he achieved the formation of microscopic structures which he claimed were somewhat similar to cells, due to their growth, motility and osmotic properties. These structures were promptly divided into two major groups: the so-called *colpoids*, which were produced when olive oil, gasoline and other complex molecules were used, and the *sulphobes*, which resulted from the mixture of NH_4SCN and H_2CO (Herrera 1942). The allotropic states of sulfur, which include complex, leaf-like structures, convinced Herrera that this morphogenetic process was related to the emergence of biological order (Herrera 1942). After many trials, Herrera found that the best starting material for the formation of his sulphobes was ammonium thiocyanate, which he dissolved in formaline (i.e. an aqueous solution of formaldehyde) and spread in thin layers until evaporation (Herrera 1942). In a number of papers he argued that similar processes could have taken place from cyanides present in the early Earth which had reacted with volcanic sulfur. As reported by Herrera in one of his most well-known papers published in *Science* in 1942 (Herrera 1942), the reactions of these precursors gave rise not only to several kinds of cell-like microstructures, but also to starch, two uncharacterized amino acids, globules of

red, green and yellow pigments, as well as what he described as a 'proteinoid condensation product'.

Apart from the historical interest, Herrera's work has caught the attention of several authors (Smith *et al.* 1968; Kenyon & Steinman 1969; Kenyon 1984) due to the potential prebiotic relevance of the manifold experiments he performed. Based on old observations by Gautier (1901, 1910), who suggested that NH_4SCN was formed from volcanic gases, Steinman *et al.* (1968) reported the synthesis of methionine by the ultraviolet (UV) irradiation of an aqueous solution of ammonium thiocyanate. However, Van Trump & Miller (1972) could not confirm these results. Comparable experiments to which glycine and several salts were added lead to the formation of insoluble microspheres endowed with peroxidase activity (Smith *et al.* 1968, 1971). Hydrolysis of an oligomer formed by the neutron beam irradiation of an aqueous solution of ammonium thiocyanate produced several amino acids, although no sulfur-containing amino acids were detected among them (Nakamura & Koga 1982).

Ammonium thiocyanate may have played a significant role in prebiotic chemistry. Its tautomer (H—N=C=S) has been reported in interstellar clouds (Irvine 1998), and it has been suggested that ammonium thiocyanate is formed in hydrothermal vents by the reaction of HCN with sulfur (Mukhin 1974; Dowler & Ingmanson 1979). Thiocyanate can also be formed by the reactions of molybdenum-sulfur compounds with cyanide under primitive Earth conditions (Mitchell & Pygall 1979). Ammonium thiocyanate, thiourea and thioacetamide have been synthesized by sparking a gaseous mixture of NH_3, CH_4, H_2O and H_2S (Heyns *et al.* 1957). Since there are relatively few reports of the synthesis of sulfur-containing amino acids under prebiotic conditions (Choughuley & Lemmon 1966; Steinman *et al.* 1968; Khare & Sagan 1971; Van Trump & Miller 1972; Getoff & Schenck 1982), thiocyanate could be a potential precursor for the abiotic synthesis of methionine and cysteine.

In the present work, we report the outcome of several experiments in which some of Herrera's studies performed in 1933, and which he continued until his death in 1942, were reproduced. As shown by photocopies of some of his laboratory notes (available upon request) by 1933 Herrera believed he had achieved the synthesis of glycine, cysteine and cystine. These compounds, which Herrera synthesized using formaldehyde and ammonium thiocyanate as starting materials, were based on the glycine synthesis from formaldehyde and potassium cyanide reported by Klages (1903) and Ling & Nanji (1922). Although in his 1942 paper Herrera mentioned the synthesis of 'starch, at least two amino acids, a condensation product of protein character and globules of green, yellow, and red pigments' (Herrera 1942), he did not list the amino acids he reported nor characterize the other products he obtained. Here we attempt to do so, based on the repetition of some of these experiments and the use of modern analytical tools. The detection of the reaction products was achieved by the employment of analytic techniques, such as high-pressure liquid chromatography (HPLC), size exclusion separation methods and infrared (IR) spectroscopy. Our results confirm the synthesis of amino acids reported by Herrera in 1942.

Table 1. *Physical properties of the precipitates formed in the reaction mixture*

PP	Weight (g)	Colour	Melting point (°C)	Comments
1	24.92	Yellow	160–185	Not pure, with yellow and white crystals
2	4.05	Yellow	155–190	
3	0.32	White	190–260	
3	0.30	Yellow	115–135	

Materials and methods

Thermal condensation experiments with ammonium thiocyanate

We first mixed 20 ml of 37% formaldehyde (0.05 M final) with 36 g of ammonium chloride (0.067 M final) and put this solution into a three-mouth flask that was kept on ice. The mixture was stirred all the time with a mechanical stirrer. We then prepared a 0.055 M ammonium thiocyanate solution that was poured for 30 minutes with a separator funnel. When half of the thiocyanate solution was added, 25 ml of glacial acetic acid was dropped and the solution was stirred for two more hours. The temperature was always kept below 10 °C. A precipitate was formed with yellow and white crystals. The mixture was filtered and the crystals were air-dried. By a fractionated precipitation (see Table 1), we obtained two more precipitates (the second one contained yellow crystals, and the third one yellow and white crystals that were separated). Based on their IR spectra, we decided to work with precipitate 1 and precipitate 3 (white crystals only). These were analysed by IR spectroscopy, a size exclusion separation method and HPLC.

Complementary experiments with some components of the reaction mixture

In order to characterize separately the products formed in the reaction mixture, we mixed some of the starting materials and we characterized them by IR spectroscopy.

(A) Synthesis of isoperthiocyanic acid

Isoperthiocyanic acid was prepared as described by Newman (1975). A concentrated solution of ammonium thiocyanate was mixed with 50% sulfuric acid and kept on ice. Almost immediately, the solution turned yellow and a precipitate of the same colour was formed.

(B) Synthesis of isoperthiocyanic acid from the reagents in Herrera's experiments

We mixed ammonium thiocyanate and formaldehyde 1:1. The mixture turned yellow and yellow crystals precipitated immediately. This compound did not show the C=O band at 1760 cm^{-1}, nor the C—H vibration at 2850 cm^{-1} from the

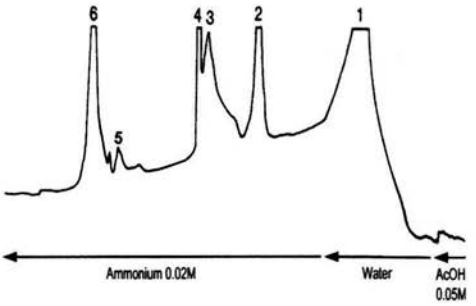


Fig. 1. Size exclusion separation of products from precipitate 1, using a SP-Sephadex cm-25 column. Peak 1 corresponds to the thiocyanate ion that did not react. Peaks 2 to 6 were analysed separately for amino acid content.

aldehyde. The IR data is suggestive of a mixture of isoperthiocyanic acid and an additional compound with formaldehyde.

(C) Synthesis of isodithiocyanic acid

Five grams of ammonium thiocyanate and 7 ml of acetic anhydride were refluxed for one hour. The mixture was put into cold water and brown crystals precipitated. These were dried and their IR spectra were obtained. The IR spectrum showed a band at 3180 cm^{-1} corresponding to the N—H vibration, a band at 2049 cm^{-1} corresponding to the C≡N vibration, a band at 1542 cm^{-1} corresponding to the C=N vibration and a band at 1131 cm^{-1} corresponding to the C=S vibration. This spectrum matches that of isodithiocyanic acid.

(D) Synthesis of isodithiocyanic acid from the reagents in Herrera's experiments

Ammonium thiocyanate and acetic acid were mixed in the same proportions as the previous experiment and the same procedure followed. The IR spectra of the resulting compound had the same bands as the precipitate formed with the mixture of ammonium thiocyanate and acetic anhydride, which corresponds to isodithiocyanic acid.

Size exclusion separation method

We purified 1 g of precipitate 1 (in two runs of 500 mg each) and 90 mg of precipitate 3 on a SP-SEPHADEX CM-25 column. Several fractions were obtained from each precipitate (Fig. 1). Each of these fractions was analysed for the presence of glycine, ammonium thiocyanate and formaldehyde (Welcher 1963; Stahl 1969; Feigl 1983). These fractions were evaporated to approximately 1 ml and lyophilized for their HPLC analysis.

HPLC chromatography

HPLC chromatography was performed at the Amino Acids Unit of the Instituto de Investigaciones Biomédicas, UNAM. The samples were analysed in a Beckman Instruments HPLC series chromatograph. The column used was C_{18} with a particle size of 5 µm. The analyses were carried out at room temperature at a wavelength of 340 nm.

The retention time of various amino acids was determined using a Beckman reference mixture. The fractions of precipitates 1 and 3 were hydrolyzed with sequential grade HCl (Pierce), acetic acid for HPLC and HPLC grade water, derivatized with ortho-phthaldialdehyde (Lindroth & Moppen 1979) and analysed for the presence of amino acids (Ladrón de Guevara *et al.* 1985).

Results

IR spectra

The characteristics of the IR spectra from the different precipitates showed a very intense band at 3140 cm^{-1}, the stretching frequency for the N—H vibration with shoulders at 2800 cm^{-1}, and a band at 2050 cm^{-1} that was assigned to the S—C≡N group. This band indicates that thiocyanate ion still persists. A small band in the region of 1700–1600 cm^{-1} corresponds to the C=O group. Another very intense band present in all the samples at 1400 cm^{-1} is due to the C=N vibration.

All these bands seem to indicate that the yellow crystals of precipitates 1 and 3 correspond to the same compound. Hence, the bands N—H at 3190 cm^{-1}, C≡N at 1515 cm^{-1}, C=S at 1006 cm^{-1} and C=O at 1667 cm^{-1} can be attributed to isoperthiocyanic acid. After acid hydrolysis, the IR spectra of all precipitates showed an increase in the C=O band at 1630 cm^{-1}.

Analysis of the IR spectra of the precipitates from the complementary experiments suggests that a fraction of the yellow precipitate observed in the reaction mixture of ammonium thiocyanate and formaldehyde corresponds to isoperthiocyanic acid. Isodithiocyanic acid was obtained when ammonium thiocyanate reacted with either acetic anhydride or acetic acid.

Amino acid analysis by HPLC

In order to identify the amino acids that could be formed in the reaction mixture, the white crystals of precipitates 1 (in two runs of 500 mg each) and 90 mg of precipitate 3 were first purified by a size exclusion separation method using a SP-Sephadex cm-25 column. We obtained five to six peaks from each precipitate. The first one, which eluted with water, corresponds to the thiocyanate ion that did not react. Several fractions were obtained from each precipitate (Fig. 1). As described above, each of these fractions was analysed for the presence of amino acids, ammonium thiocyanate and formaldehyde. These fractions were evaporated to approximately 1 ml and lyophilized for their HPLC analysis. The results of the analyses of peaks 2 of precipitates 1 and 3 are shown in Table 2.

The HPLC chromatograms presented here suggest the presence of several compounds whose retention times correspond to glycine, alanine, cysteine and methionine. From these, only cysteine and methionine are sulfur-containing

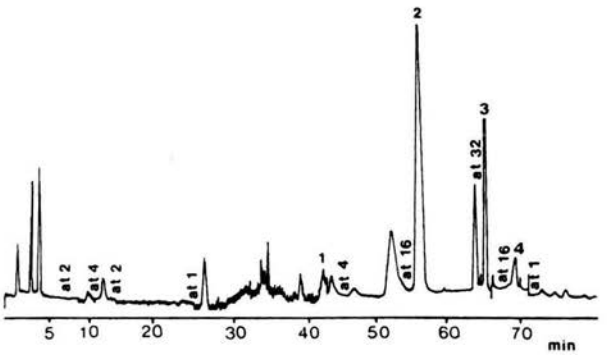


Fig. 2. HPLC identification of: 1, glycine; 2, alanine; 3, cysteine; 4, methionine. Amino acids were identified by their retention times as described in the text.

Table 2. *Amino acid yield quantified by HPLC. Concentration = nmol mg^{-1} of sample. The first four runs correspond to precipitate 1 and the next four to precipitate 3. ND represents 'Not determined'*

Run No.	Glycine	Alanine	Methionine	Cysteine
1	19	315	22	109
2	3	495	74	ND
3	Traces	627	27	191
4	Traces	192	29	215
5	4	259	1	132
6	3	62	4	23
7	63	642	22	288
8	20	205	23	91

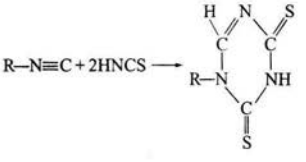
amino acids. Although we analysed standard samples of other sulfur-containing amino acids, such as taurine and cystine, the peaks obtained in the chromatograms do not correspond to their retention times (Fig. 2). Other unidentified peaks in the chromatogram could correspond to non-proteinic amino acids. The amino acid formation yield was low and was determined by the electronic integration of the peak area (Table 2).

The same amino acids were observed in both the hydrolyzed and non-hydrolyzed fractions, although the yield was higher in the former ones.

Discussion and conclusions

Like many of his contemporaries, Herrera was convinced that the first living beings had been autotrophic organisms. The popularity of Pflüger's (1875) ideas on biological catalysis had convinced him that CN derivatives played an essential role in biochemical processes and, hence, that cyanogen and its derivatives must have been present at the origin of life. Led by the schemes which suggested at the time that H_2CO was a central intermediate in the photosynthetic fixation of CO_2, Herrera attempted the laboratory formation of an autotrophic protoplasm by mixing the precursors he felt were essential for a minimal living system. It was not because of foresight that he employed compounds which are nowadays recognized as potential components of the prebiotic environment. Instead, he should be recognized as a careful worker, whose deep knowledge of the major theories of his contemporaries led him to study the origins of life within the framework of his times.

Because of the assumption of primordial photosynthesis, formaldehyde was one of the main components in Herrera's sulfocyanic theory. It is generally accepted that H_2CO was present in the primitive Earth (Pinto & Gladstone 1980; Wills & Bada 2000), and ammonium thiocyanate is produced in hydrothermal vents (Mukhin 1974; Dowler & Ingmanson 1979). The thiocyanate ion is a highly reactive chemical species that can give rise to a great variety of compounds that could be intermediates for the synthesis of various heterocycles such as imidazoles, purines, thiazoles and pyrimidines (Gompper *et al.* 1966), albeit under conditions that are not prebiotic. Isothiocyanic acid (HNCS) has been detected in the interstellar medium (Irvine 1998). The reaction of isothiocyanic acid with isonitriles can lead to triazines (Ugi *et al.* 1965).

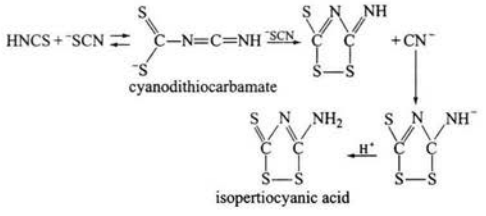


Condensation of ammonium thiocyanate is known to give rise to a yellow-red polymer (Hall & Wilson 1969), which may explain the colours described by Herrera (1942). In solution, ammonium thiocyanate participates in the following equilibrium:

$$NH_4SCN \rightleftarrows NH_3 + HSCN$$

Simultaneously, thiocyanic acid is in equilibrium with its tautomer HNCS, which in a gaseous state and in aqueous and

non-aqueous solutions is more abundant than thiocyanic acid (Newman 1975). Isothiocyanic acid is mainly found in its non-dissociated form, and in solution polymerizes rapidly to form isoperthiocyanic acid, a compound with an intense yellow colour (Hall & Wilson 1969). Isoperthiocyanic acid is produced directly from thiocyanate in aqueous solution, and probably corresponds to the 'yellow pigment' described by Herrera (1942). Our observations also confirm the presence of this heterocycle as the most abundant product in our experiments.



Additional products may be formed from the highly reactive mixture described by Herrera (1942). In principle, thiourea could also be formed, as a result of the isomerization of thiocyanate:

$$NH_4SCN \rightleftharpoons H_2NCS{-}NH_2$$

or by addition of ammonia to the CN bond, in the isothiocyanic acid:

$$H{-}N{=}C{=}S + NH_3 \rightarrow H_2NCS{-}NH_2$$

However, we have not searched for these products.

Herrera (1942) did not characterize the amino acids he synthesized. As he was following the procedure described by Klages (1903) and Ling & Nanji (1922),

$$2HCHO + NH_4CN \rightarrow CH_2NCH_2CN + 2H_2O$$

$$CH_2NCH_2COOH + H_2O \rightarrow CH_2(NH_2)COOH + HCHO$$

Herrera was performing a variation of a Strecker synthesis, in which ammonium thiocyanate could take the place of NH_4CN and where the hydrolysis of the nitrile was achieved by boiling with a highly concentrated solution of barium hydroxide (Ling & Nanji 1922).

Although in his laboratory notes Herrera reports the synthesis of cysteine, in his 1942 *Science* paper he does not characterize the amino acids he obtained. Did he achieve the synthesis of methionine? It is unfortunate that only rather crude analytical methods were available to Herrera. The preliminary results presented here suggest that, in the experiments which we have performed following the procedures described by Herrera, four amino acids were synthesized: glycine, alanine, cysteine and methionine, with a yield of 2% (Fig. 2). Alanine was the most abundant amino acid in all the samples analysed. It is possible that some of the peaks that we could not identify by HPLC correspond to other non-proteinic amino acids, such as β-amino isubutyric acid and thioglycine.

Relatively few efforts have been devoted to the prebiotic synthesis of sulfur-containing amino acids. A 1% yield abiotic synthesis of methionine was first reported by Steinman *et al.* (1968), who UV-irradiated a 0.1 M aqueous solution of ammonium thiocyanate, but their results have not been confirmed by others (Van Trump & Miller 1972). Until now, the only solid report of methionine synthesis involves the Strecker reaction sparking of a mixture of CH_4, N_2, H_2O and traces of NH_3, to which H_2S was added (Van Trump & Miller 1972).

The preliminary results presented here suggest that a variation of the Strecker synthesis involving formaldehyde and ammonium thiocyanate could lead to low yields of amino acids, including sulfur-containing amino acids. Alternatively, it is possible that the amino acids reported here, including cysteine and methionine, are the outcome of an hydrolysis of oligomers that could be formed by the self-condensation of SCN, by a mechanism equivalent to that reported by Ferris *et al.* (1978). These possibilities should be re-evaluated once the identification of all the products reported here is confirmed by other analytical methods, such as X-ray powder diffraction and mass spectrometry, and kinetic studies on the stability of synthesis and degradation of these compounds under the conditions described here are performed. Work now in progress will address these issues. Nevertheless, our results suggest that the use of ammonium thiocyanate, advocated by Herrera (1942) in order to explain the autotrophic emergence of life, may help explain the accumulation of methionine and cysteine under primitive Earth conditions as required by a heterotrophic origin of life.

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MOLECULAR BIOLOGY AND THE RECONSTRUCTION OF MICROBIAL PHYLOGENIES: DES LIAISONS DANGEREUSES?

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1. INTRODUCTION

Only forty years after the DNA double helix model was first suggested, molecular biology has become one of the most provocative, rapidly developing fields of of scientific research, that has led not only to tantalizing new findings on processes and mechanisms at the molecular level, but also to major conceptual revolutions in life sciences. Is there any hope of developing methodological approaches and theoretical frameworks not only to make sense of the overwhelming growing body of data that this relatively new field is producing, but also to use them to develop a more integrative, truly multidisciplinary understanding of biological phenomena? As Peter Bowler wrote a few years ago, Charles Darwin and his followers were accutely aware that "evolutionism's strength as a theory came fom its ability to make sense out of a vast range of otherwise meaningless facts" (Bowler, 1990). This situation has not changed. Attempts to explain the basic properties of the living beings as the result of complex systems in whose emergence self-organization processes have played a major role are now fashionable (Kauffman, 1993). However, the unifying powers of evolutionary biology have not been diminished. In fact, they probably represent one of the most promising possibilities of overcoming the perils of reductionism that have plagued molecular biology since its inception.

Molecular approaches to evolutionary issues are a century old. The possibility of developing a sucessful blending between them may have

been first suggested by the American-born British biologist and physician George H. F. Nuttall, who in 1904 published a book summarizing the results of the detailed comparisons of blood proteins that he had used to reconstruct the evolutionary relationships of animals. "In the absence of palaeontological evidence", wrote Nuttall (1904), "the question of the interrelation-ship amongst animals is based upon similarities of structure in existing forms. In judging of theses similarities, the subjective element may largely enter, in evidence of which we need but look at the history of the classification of the Primates." Such subjective element, Nuttall believed, could be successfully overcomed by constructing a phylogeny based not on form but on the inmunological reactions of blood-related proteins.

Although the comparative analysis of biochemical properties, metabolic pathways and, in few cases, morphological characteristics, had provided some useful insights on the evolutionary relationships among certain microorganisms, until a few years ago the reconstruction of bacterial phylogenies and the understanding of microbial taxonomy were both viewed with considerable skepticism. This situation has undergone dramatic changes with the recognition that proteins and nucleic acid sequences are historical documents of unsurpassed evolutionary significance (Zuckerkandl and Pauling, 1965), and has led to a radical renovation of the phylogeny, classification, and systematics of prokaryotic and eukaryotic microbes (Woese, 1987).

But these changes have also sparked new debates, and have led to an increased appreciation that the scope and limits of molecular cladistic methodologies require clarification. As shown by the current controversies on the characteristics of the first organisms, the origin of the different components of the eukaryotic cell, the tempo and mode of microbial evolution, and the soundness of traditional taxonomic systems, the development of the full potential of molecular cladistics will depend not only on methodological refinements to improve the algorithms used for reconstructing evolutionary history from molecular data, but also the critical reexamination of its theoretical framework, which includes a number of central concepts, most of which were grafted from classical evolutionary theory into molecular biology. The purpose of this chapter is to discuss some of these issues, and to review briefly some of the major contributions that they have promoted in our understanding of previously uncharacterized early periods of biological evolution.

2.A EUKARYOTE IS A EUKARYOTE IS A EUKARYOTE?

The awareness that genomes are extraordinarily rich historical documents from which a wealth of evolutionary information can be retrieved has widened the range of phylogenetic studies to previously unsuspected heights. The development of rapid nucleic acid sequencing techniques, combined with the simultaneous and independent blossoming of computer science, has led not only to an explosive growth of sequence databases and new sophisticated tools for their exploitation, but also to the recognition that different macromolecules may be uniquely suited as a molecular chronometers in the construction of nearly universal phylogenies.

A major achievement of this approach has been the evolutionary comparison of small subunit ribosomal RNA (rRNA) sequences, which has allowed the construction of a trifurcated, unrooted tree in which all known organisms can be grouped in one of three major (apparently) monophyletic cell lineages: the eubacteria, the archaebacteria, and the eukaryotic nucleocytoplasm, now referred to as new taxonomic categories, i.e., the domains *Bacteria*, *Archaea*, and *Eucarya*, respectively (Woese et al., 1990). There is strong evidence that the identification of these lineages is not an artifact based solely upon the reductionist extrapolation of information derived from one single molecule. It is true that the congruence between rRNA genes and other molecules is not always ideal, and anomalous phylogenies have been reported (Rivera and Lake, 1992; Tiboni et al., 1993; Gupta and Golding, 1993). At the time being there is no general explanation to account for these preculiar topologies, and the possibility that we may have to restrict ourselves to empirical characterizations of such cases should be kept in mind. However, a large variety of phylogenetic trees constructed from DNA and RNA polymerases, elongation factors, F-type ATPase subunits, heat-shock and ribosomal proteins, and an increasingly large set of genes encoding enzymes involved in biosynthetic pathways, have confirmed the existence of the three primary cellular lines of evolutionary descent (Doolittle and Brown, 1994).

The ensuing tripartite taxonomic description of the living world fostered by C. R. Woese and his followers has been disputed by a number of workers, who contend that both eubacteria and archaebacteria are *bona fide* prokaryotes, regardless of the pecularities that separate that separate them at the molecular level (Mayr, 1990; Margulis and Guerrero, 1991; Cavalier-Smith, 1992). Furthermore, because of their very nature,

molecular dichotomous phylogenetic trees cannot be drawn which include anastomozing branches corresponding to the lineages which gave rise to the different components of eukayotic cells. Accordingly, Margulis and Guerrero (1991) have argued that although molecular cladistics is now a prime force in systematics, phylogenetically accurate taxonomic classifications should be based not only on the evolutionary comparison of macromolecules, but also on metabolic pathways, chromosomal cytology, ultrastructural morphology, biochemical data, life cycles, and, when available, paleontological and geochemical evidence.

By showing the evolutionary proximity of mitochondria and chloroplasts to purple bacteria and cyanobacteria, respectively, molecular phylogenies have confirmed the endosymbiotic theory. Moreover, a number of trees also suggest that a major portion of the eukaryotic nucleocytoplasm originated from an archaebacteria-like cell whose descendants form the monophyletic eucaryal branch (Gogarten-Boekels and Gogarten, 1994). As asserted by Woese and his collaborators, although the presence of endosymbionts is of critical importance to the eukaryotes, it is undeniable that the latter "have a unique, meaningful phylogeny" (Wheelis et al., 1992). While such view assumes an absolute continuity between the nucleocytoplasm and its direct ancestor, the holistic arguments advocated by Margulis and Guerrero (1991), Cavalier-Smith (1992), and others, emphasize the evolutionary emergence of an novel type of cell as a result of endosymbiotic events. According to the latter, the key transitional event leading to nucleated cells was the evolutionary acquisition of heritable intracellular symbionts, and the eucaryal branch does not represent eukaryotic cells as a whole, any more than fungal hyphae or phycobionts like the *Trebouxia* algal cells exhibit, by themselves, all the phenotypic and genetic characteristics of a lichen thallus.

Of course, antagonistic taxonomies have coexisted more or less peacefully along the history of biology. However, the urgent need to critically revise current classificatory systems cannot be underscored. Modern taxonomic schemes need to acknowledge not only the existence of three major cell lineages, but also the eukaryotic divergence patterns, which appear to be the result of rapid bursts of speciation (Sogin, 1994). Any such modifications in biological classification require the recognition of the functional and anatomical continuity between the eukaryotic cytoplasm and the intranuclear environment, as well as the likelihood that the evolution of membrane-bounded nuclei is indeed a byproduct of permanent intracellular associations. In fact, even

amitochondrial eukaryotes such as *Giardia* and *Trychomonas* appear to have permanent intracellular bacterial endosymbionts (Nemanic et al., 1979; Feely et al., 1988). These pre-mitochondrial cells, which may include the microaerophilic, amitotic, multinucleated giant amoeba *Pelomyxa palustris*, are all located in the oldest branches of the eucarya, and contain several types of intracellular prokaryotes which may be the functional equivalents of mitochondria. The ubiquity of endosymbionts suggests that they may have played a critical role in the evolutionary development of nucleated cells. This hypothesis is amenable to observational and experimental designs, and may be supported by studying the possible bacterial affinities of membrane-bounded hydrogenosomes that are known to multiply by binary division in the *Trychomonas* cytoplasm (Müller, 1988), as well as by searching for prokaryotic endosymbionts in species of Parabasalia, Retortomonads, Diplomonads, Calonymphids, and other protist taxa which apparently evolved prior to mitochondrial acquisition.

3. THE ROOT OF THE TREE OR THE TIP OF THE TRUNK?

The construction of the unrooted rRNA tree showed that no single major branch predates the other two, and all three derive from a common ancestor. It was thus concluded that the latter was a progenote, which was defined as a hypothetical entity in which phenotype and genotype still had an imprecise, rudimentary linkage relationship (Woese and Fox, 1977). According to this view, the differences found among the transcriptional and translational machineries of eubacteria, archaebacteria, and eukaryotes, were the result of evolutionary refinements that took place separately in each of these primary banches of descent after they have diverged from their universal ancestor (Woese, 1987).

From an evolutionary point of view it is reasonable to assume that at some point in time the ancestors of all forms of life must have been less complex than even the simpler extant cells, but our current knowledge of the characteristics shared between the three lines has shown that the conclusion that the last common ancestor was a progenote was premature. This interpretation, based on rRNA-based trees for which no outgroups have been discovered, has been definitively superseded (Woese, 1993). A partial description of the last common ancestor of eubacteria, archaebacteria, and eukaryotes may be inferred from the distribution of homologous traits among its descendants. The set of

such genes that have been sequenced and compared is still small, but the sketchy picture that has already emerged suggests that the most recent common ancestor of all extant organisms, or *cenancestor*, as defined by Fitch and Upper (1987), was a rather sophisticated cell with at least (a) DNA polymerases endowed with proof-reading activity; (b) ribosome-mediated translation apparatus with an oligomeric RNA polymerase; (c) membrane-associated ATP production; (d) signalling molecules such as cAMP and insulin-like peptides; (e) RNA processing enzymes; and (f) biosynthetic pathways leading to amino acids, purines, pyrimidines, coenzymes, and other key molecules in metabolism (Lazcano et al., 1992; Benner et al., 1993).

These traits are far to numerous and complex to assume that they evolved independently or that they are the result of massive multidirectional horizontal transfer events which took place before the earliest speciation events recorded in each of the three lineages. Their presence suggests that the cenancestor was not a direct, immediate descendant of the RNA world, a protocell or any other pre-life progenitor system. Very likely, it was already a complex organism, much akin to extant bacteria, and must be considered the last of a long line of simpler earlier cells for which no modern equivalent is known.

Unfortunately, the characteristics of evolutionary predecessors of the cenancestor cannot be inferred from the plesiomorphic traits found in the space defined by rRNA sequences. Although trees constructed from such universally shared characters appear to be free of internal inconsistencies, the lack of outgroups leads to topologies that specify branching relationships but not the position of the ancestral phenotype. Thus, such trees cannot be rooted. This phylogenetic *cul-de-sac* may be overcomed by using paralogous genes, which are sequences that diverge not through speciation but after a duplication event. As noted almost twenty years ago by Schwartz and Dayhoff (1978), rooted trees can be constructed by using one set of paralogous genes as an outgroup for the other set, a rate-independent cladistic methodology that expands the monophyletic grouping of the sequences under comparison.

This approach was used independently a few years ago by Iwabe et al (1989) and Gogarten et al (1989), who analyzed paralogous genes encoding (a) the two elongation factors (EF-G and EF-Tu) that assist in protein biosynthesis; and (b) the alpha and beta hydrophilic subunits of F-type ATP synthetases. Using different tree-constructing algorithms, both teams independently placed the root of the universal trees between the eubacteria, on the one side, and archaebacteria and eukaryotes on the

other. Their results imply that eubacteria are the oldest recognizable cellular phenotype, and suggests that specific phylogenetic affinities exist between the archaea and the eucarya.

This branching order, which was promptly adopted by Woese et al (1990), appears to be consistent with structural and functional similarities which are known to exist in the translation and replication machineries of both archaebacteria and eukaryotes (Kletzin, 1992; Ouzonis and Sander, 1992; Slesarev et al., 1993; Kaine et al., 1994). However, the issue is far from solved. No support for a particular topology was detected when mean interdomain distance analysis was used to analize a set of approximately forty genes common to the three lineages (Doolittle and Brown, 1994). The situation is further aggravated by the fact that the phylogenetic analysis of sets of ancestral paralogous genes other than the elongation factors and the ATPase hydrophilic subunits has challenged the conclusion that universal trees are rooted in the eubacterial branch (Benahenchou-Lahfa et al., 1993; Gupta and Singh, 1992; Forterre et al., 1993). While the products of genes involved in the transcription/translational molecular machinery of eukaryotes appear to be closer to those of archaebacteria than to eubacteria, other sequences such as those encoding heat-shock proteins and several enzymes suggest the existence of phylogenetic affinities between archaea and Gram positive bacteria. These observations have led to the suggestion that the pre-eukaryotic host was the outcome of a fussion event between eubacteria and archaebacteria, that took place prior to the endosymbiotic acquisition of mitochodnria and chloroplasts (Gupta, 1995).

The lack of congruency between different universal phylogenies may be the result not only of the statistical problems involved in the aligment and comparison of a large number of sequences that may have diverged more than 3.5×10^9 years ago, but also of even older additional paralogous duplications (Forterre et al., 1993), and of horizontal gene tranfer events (Hilario and Gogarten, 1993), both of which may be obscuring the natural relationships between the lineages. Given the likelihood that microbial phylogenetic analysis will increase its reliance on paralogous duplicates to define outgroups and character polarities (Sidow and Bowman, 1991), detailed studies should be devoted to assess the validity and limits of this cladistic methodology.

Minor differences in the basic molecular processes of the three main cell lines can be distinguished, but all known organisms, including the oldest ones, share the same essential features of genome replication,

gene expression, basic anabolic reactions, and membrane-associated ATPase mediated energy production. The molecular details of these universal processes not only provide direct evidence of the monophyletic origin of all extant forms of life, but also imply that that sets of genes encoding the components of these complex traits were frozen a long time ago, i. e., major changes in them are very strongly selected against and are lethal. Biological evolution prior to the divergence of the three domains was not a continuous, unbroken chain of progressive transformation steadily proceeding towards the cenancestor. However, no evolutionary intermediate stages or ancient simplified version of the basic biological processes have been discovered in extant organisms.

Nevertheless, clues to the genetic organization and biochemical complexity of the earlier entities from which the cenancestor evolved may be derived from the analysis of paralogous sequences. Their presence in the three cell lineages implies not only that their last common ancestor was a complex cell already endowed, among others, with pairs of homologous genes encoding two elongation factors, two ATPase hydrophilic subunits, two sets of glutamate dehydrogenases, and the A and B DNA polymerases, but also that the cenancestor itself must have been preceded by simpler cells in which only one copy of each of these genes existed. In other words, Archean paralogous genes provide evidence of the existence of ancient organisms in which ATPases lacked the regulatory properties of its alpha subunit, protein synthesis took place with only one elongation factor, and the enzymatic machinery involved in the replication and repair of DNA genomes had only one polymerase ancestral to both DNA polymerase I and II.

By definition, the node located at the bottom of the cladogram is the root of a phylogenetic tree, and corresponds to the common ancestor of the group under study. But names may be misleading. The recognition that basic biological processes like DNA replication, protein biosynthesis, and ATP production require today the products of pairs of genes which arose by paralogous duplications during the early Archean, implies that what we have been calling the root of universal trees is in fact the tip of a trunk of unknown length in which the history of a long (but not necessarily slow) series of archaic evolutionary events may still be recorded. The inventory of paralogous genes that duplicated during this previously unchacterized stage of biological evolution appears to include, in addition to elongation factors, ATPase subunits, and DNA polymerases, the sequences encoding heat shock proteins, ferredoxins, dehydrogenases, DNA topoisomerases, several pairs of aminoacyl-

tRNA synthetases, and enzymes involved in nitrogen metabolism and amino acid biosynthesis. It is noteworthy that this list includes also aspartate transcarbamoyl transferase, an enzyme which together with carbamyl phosphate synthetase (whose large subunit is itself the product of an internal, i.e., partial, paralogous duplication) catalyzes the initial steps of pyrimidine biosynthesis (García-Meza et al, 1995).

Thus, prior to the early duplication events that led to what may be a rather large number of cenancestral paralogous sequences, simpler living systems existed that lacked the large set of enzymes and the sophisticated regulatory abilities of contemporary cells. Although lateral transfer of coding sequences may be almost as old as life itself, gene duplication followed by divergence probably played a prominent role in the accretion of complex genomes, and may have led to a rapid rate of microbial evolution. If it is assumed that the rate of gene duplicative expansion of ancient cells was comparable to today's present values, which are of 10^{-5} to 10^{-3} gene duplications per gene per cell generation (Stark and Wahl, 1984), the maximum time required to go from an hypothetical 100 gene organism to one endowed with a filamentous cyanobacterial-like genome of approximately 7000 genes would be less than ten million years (Lazcano and Miller, 1994).

Although the are no published data on the rate of formation of new enzymatic activities resulting from gene duplication events under either neutral or positive selection conditions, the role of duplicates in the generation of evolutionary novelties is well stablished. Once a gene duplicates, one of the copies may be free to accumulate non-lethal mutations and acquire new additional properties, which could lead into its specialization or recruitment into new role. Data summarized here supports the idea that primitive biosynthetic pathways were mediated by small, inefficient enzymes of broad substrate specificity (Jensen, 1976). Larger substrate ranges may had not been a disadvantage, since relatively unspecific enzymes may have helped ancestral cells with reduced genomes overcome their limited coding abilities (Ycas, 1974).

The discovery that homologous enzymes catalyzing similar biochemical reactions are part of different anabolic pathways supports the idea that enzyme recruitment took place during the early development of several basic anabolic pathways (Fani and Lazcano, in prep.). Evolutionary tinkering of the products of duplication events apparently had a major role in metabolic evolution. This is supported by the analysis of the *Escherichia coli* sequence databases, that has shown that approximately 36% of the proteins whose sequence is available are the result of

duplication events (Riley, 1993). Such high levels of redundancy represent an illuminating possibility and suggest that the wealth of phylogenetic information older than the cenancestor may be larger than realized, and its analysis may provide fresh insights into a crucial but largely undefined stage of early biological evolution during which major biosynthetic pathways emerged and became fixed.

There is a major exception to the above conclusion. True fungi, euglenids, and chytridiomycetes synthesize lysine via an eight-step pathway in which a-aminoadipate (AAA) is an intermediate. This route is different from the seven-step diaminopimelate pathway used by bacteria, plants, and most protists (Bhattacharjee, 1985). The phylogenetic distribution of these pathways suggest that the diaminopimelate route is the most recent one. Accordingly, if the patchwork assembly of metabolic pathways (Jensen, 1976) is valid, then it can be predicted that the enzymes catalyzing the AAA-route should be homologous to those participating in other major biosynthetic pathways.

The recognition that enzyme recruitment may have played a major role in metabolic evolution leads, however, to assume some caution in phylogenetic inferences. Although in some cases metabolic pathways may be sucessfully used to assess the phylogenetic relationship of prokaryotes (DeLey, 1968; Margulis, 1993), the possibility that some of the enzymes of archaic pathways may have survived in unusual organisms (Keefe et al., 1994), or that important portions of extant metabolic routes may have been assembled by a patchwork process (Jensen, 1976), suggest that considerable prudence should be exerted when attempting to describe the physiology of truly primordial organisms by simple direct back extrapolation of extant metabolism.

4. MOLECULAR CLADISTICS AND THE ORIGIN OF LIFE: THE WEAK CONNECTION

"All the organic beings which have ever lived on this Earth", wrote Charles Darwin in the *Origin of Species*, "may be descended from some primordial form". Although the placement of the root of universal trees is a matter of debate, the development of molecular cladistics has shown that despite their overwhelming diversity and tremendous differences, all organisms are ultimately related and descend from Darwin's primordial ancestor. But what was the nature of this progenitor?

The heterotrophic hypothesis suggested by Oparin (1938) not only gave birth to a whole new field devoted to the study of the origin of life, but played a central role in shaping several influential taxonomic schemes and different bacterial phylogenies (Broda, 1975; Almassy and Dickerson, 1978; Schwartz and Dayhoff, 1978; Margulis 1993). Although the central role of glycolysis and the wide phylogenetic distribution of at least some of its molecular components are strong indications of its antiquity (Fothergill-Gilmore and Michels, 1993), it is no longer possible to support the *ad hoc* identification of putative primordial traits to assume that the first living system was a *Clostridium*-like anaerobic fermenter (Schwartz and Dayhoff, 1978; Almassy and Dickerson, 1978) or a *Mycoplasma* type of cell (Wallace and Morowitz, 1973; Razin, 1978). Like vegetation in a mangrove, the roots of universal phylogenetic trees are sumerged in the muddy waters of the prebiotic broth, but how the transition from the non-living to the living took place is still unknown.

Indeed, we are still very far from understanding the origin and attributes of the first living beings, which may have lacked even the most familiar features in extant cells. For instance, protein synthesis is such an essential characteristic of cells, that it is frequently argued that its origin should be considered synonymous with the emergence of life itself. However, the discovery of the catalytic activities of RNA molecules has led considerable support to the possibility that during early stages of biological evolution living systems were endowed with a primitive replicating and catalytic apparatus devoid of both DNA and proteins (Lazcano, 1994a). The scheme may be even more complex, since RNA itself may have been preceded by simpler genetic macromolecules lacking not only the familiar 3',5' phosphodiester backbones of nucleic acids (Joyce et al., 1987), but perhaps even today's bases (Kolb et al., 1994).

Although the discovery that L-arginine can bind in a highly selective way to catalytic introns supports the hypothesis that the genetic code emerged in an RNA world (Yarus, 1993), the ultimate origin of the codon assigments remains an unsolved problem. Molecular cladistic analysis of the components of protein synthetic machinery (ribosomal proteins, elongation factors, aminoacyl tRNA synthetases, initiation and termination factors, etc.) may provide some clues on the late evolution of the genetic apparatus. Systematic studies of complete sets of aminoacyl-tRNA synthetases from the three cell linages are still required, but the comparison of the available sequence and tertiary structures (Cusack et al., 1990; Nagel and Doolittle, 1991) have

confirmed that these keys enzymes fall into two functionally related but distinct classes that appear to have evolved independently.

Group I includes the valyl-, isoleucyl-, and the leucyl-tRNA synthetases. The topology of the available unrooted tree of isoleucyl-tRNA synthetase sequences from the three cell linaeages suggests a sisterhood relationship between the two prokaryotic domains (Doolittle and Brown, 1994) that is not consistent with the rooted trees calculated by Gogarten et al (1989) and Iwabe et al (1989). Nonetheless, the close phylogenetic relationship between the *Escherichia coli* isoleucyl-tRNA synthetase sequence, and the yeast valyl-tRNA synthetase (Jordana et al., 1987) suggests only that valine and isoleucine synthetases are the products of still another archaic set paralogous genes diverged before the cenanestor's time. Equally significant, this similarity also suggests that the ancestral synthetase may have been a non-specific enzyme unable to discriminate between valine and isoleucine.

Since valine and isoleucine are two sterically related amino acids, have similar codons, and share the same enzymes in the last four parallel reactions of their biosynthetic pathways, the phylogenetic proximity of their tRNA synthetases suggests that the concerted evolution of an entire set of genes took place, eventually leading to a less-ambiguous code. This interpretation, which is consistent with the coevolution theory of the genetic code suggested by Wong (1975), clearly fits into previous schemes suggesting that the genetic apparatus evolved from primitive inaccurate versions in which groups of chemically similar amino acids may have been activated by unspecific synthetases (Woese, 1965; Fitch and Upper, 1987). It is likely that the hydrolytic sites that provide these enzymes with an additional sieve that allows them to discriminate between valine and isoleucine (Norris and Berg, 1964), emerged after the evolution of valyl- and isoleucyl-tRNA synthetase specificities as a result of duplication events.

Although molecular cladistics may provide clues to some late steps in the development of the genetic code, it is difficult to see how the applicability of this approach can be extended beyond a threshold that corresponds to a period of cellular evolution in which protein biosynthesis was already in operation. Older stages are not yet amenable to molecular phylogenetic analysis. Although there have been considerable advances in the understanding of chemical processes that may have taken place before the emergence of the first living systems, life's beginnings are still shrouded in mystery. A cladistic approach to this problem is not feasible, since all possible intermediates that may

have once existed have long since vanished. The temptation to do otherwise is best resisted. Given the huge gap existing in current descriptions of the evolutionary transition between the prebiotic synthesis of biochemical compounds and the cenancestor (Lazcano, 1994b), it is naive to attempt to describe the origin of life and the nature of the first living systems from the available rooted phylogenetic trees.

Nevertheless, there have been several recent attempts to use macromolecular data to support claims on the hyperthermophily of the first living organisms and the idea of a hot origin of life. The examination of the prokaryotic branches of unrooted rRNA trees had already suggested that the ancestors of both eubacteria and archaebacteria were extreme thermophiles, i.e., organisms that grow optimally at temperatures in the range 90° C and above (Achenbach-Richter et al., 1987). Rooted universal phylogenies appear to confirm this possibility, since heat-loving bacteria occupy short branches in the basal portion of molecular cladograms (Stetter, 1994).

Such correlation between hyperthermophily and primitiveness has led support to the idea that heat-loving lifestyles are relics from early Archean high-temperature regimes that may have resulted from a severe impact regime (Sleep et al., 1989). It has also been interpreted as evidence of a high temperature origin of life, which according to these hypotheses took place in extreme environments such as those found today in deep-sea vents (Holm, 1992), or in other sites in which mineral surfaces may have fueled the appearance of primordial chemoautolithotrophic biological systems (Wächtershäuser, 1990).

Such ideas are not totally without precedent. The possibility that the first heterotrophs may have evolved in a sizzling-hot environment is in fact an old suggestion (Harvey, 1924). Despite their long genealogy, these hypotheses have not been able to bypass the problem of the chemical decomposition faced by amino acids, RNA, and other thermolabile molecules which have very short lifetimes under such extreme conditions (Miller and Bada, 1988). Although no mesophilic organisms older than heat-loving bacteria have been discovered, it is possible that hyperthermophily is a secondary adaptation that evolved in early geological times (Sleep et al., 1989; Confalonieri et al., 1993; Lazcano, 1993). In fact, hyperthermophiles not only share the same basic features of the molecular machinery of all other forms of life; they also require a number of specific biochemical adaptations. Any theory on the hot origin of life must address the question of how such traits, or their evolutionary precedessors, arose spontaneously in the prebiotic

environment. Such adaptations may include histone-like proteins, RNA modificating enzymes, and reverse gyrase, a peculiar ATP-dependent enzyme that twists DNA into a positive supercoiled conformation (Segerer et al., 1993; Confalonieri et al., 1993). Clues to the origin of hyperthermophily may be hidden in this list, and its evolutionary analysis may contribute to the understanding of the rather surprising phylogenetic distribution of the immediate mesophilic descendants of heat-loving prokaryotes, which shows that at least five independent abandonments events of hyperthermophilic traits took place in widely separated branches of universal trees, one of which corresponds to the eukaryotic nucleocytoplasm (García-Meza et al., 1995).

The antiquity of hyperthermophiles appears to be well stablished, but there is no evidence that they have a primitive molecular genetic apparatus. Thus, the most basic questions pertaining to the origin of life relate to much simpler replicating entities predating by a long series of evolutionary events the oldest recognizable heat-loving bacteria. Why hyperthermophiles are located at the base of universal trees is still an open question, but the possibility that adaptation to extreme environments is part of the evolutionary innovations that appeared in trunk of the tree cannot be entirely dismissed. The phylogenetic distribution of heat-loving bacteria is no evidence by itself of a hot origin of life, any more than the presence in the hyperthermophile archaeon *Sulfolobus solfataricus* of a gene encoding a thermostable B-type DNA polymerase endowed with 3'-5' exonuclease activity (Pisani et al., 1992) can be interpreted to imply that the first living organism had a DNA genome.

5. THE TIMESCALES OF MICROBIAL EVOLUTION

Because of their short generation times and to their tendency to engage in taxonomically promiscuous lateral transfer of genes, prokaryotes can adapt rapidly to new environmental conditions and evolve at an extremely quick pace. New traits can become fixed in bacterial populations in timescales that are mere instants from a geological perspective. However, due perhaps to the extended intellectual prejudice that all evolutionary changes must proceed a in a step-wise, slow pace, it has been generally been assumed that the origin and early evolution of life were extremely slow processes requiring several billions of years (Oparin, 1938; Wald, 1954; Simpson, 1964; Cloud, 1968).

This view is no longer tenable. Late accretion impacts may have killed off life on our planet as late as 3.8 x 10^9 years ago (Sleep et al., 1989), but as shown by the Northwestern Australian fossil assemblages, an abundant, complex and highly diversified microbiota which may have included stromatolite-building cyanobacteria, was fluorishing only 300 million years after the period of intense bombardment had ended (Schopf, 1993). Since rRNA-based phylogenetic trees suggest that cyanobacteria are a late eubacterial clade (Woese, 1987), Archean life must have rapidly achieved levels of genetic organization, biochemical complexity, ecological diversity, and evolutionary potential, comparable to those of extant bacterial populations.

Although it is not possible to assign a precise chronology to the earliest branching events, there are several indications that the three domains of life could have already been distinct by the early Precambrian. The fact that the inside of the cell nucleus is a poorly oxygenated milieu in which no O_2-dependent metabolic pathways appear to exist (Joenje, 1989), may be interpreted as the retention of a primitive feature, thereby supporting the idea that the ancestor of the eukaryotic nucleocytoplasm branched off early in the history of planet. Of course, no mitochondrial-bearing eukaryotes could have survived in the oxygen-poor primitive environment, but the free-living ancestors of parasitic amitochondrial anaerobes like *Giardia* could have done so (Knoll, 1992).

While it is true that for many groups of plants and animals there is a remarkably well-established correlation between the number of amino acid differences and the time since their evolutionary divergence from a common ancestor, it is notoriously difficult to put microbial molecular evolution within a temporal framework. Base-pair substitution rates estimated from paleontological information have been used to calibrate molecular trees, but the prokaryotic fossil record is so scanty that estimates on the chronological lengths of bacterial branches and the assigment of reliable dates to phylogenetic trees are precluded.

Due to the inadequeacy of the bacterial fossil record, Ochman and Wilson (1987) attempted to obtain multiple calibration points for the eubacterial 16S rRNA lineage by linking the divergence of some of their branches with the geological record. The antiquity of the nitrogen-fixing ***Rhizobium*** and luminous ***Photobacterium*** bacteria, which have symbiotic associations with legumes and marine fishes, respectively, was estimated from the plant and vertebrate fossil record, for example. Approximately 1% base substitution per 50 million years was calculated (Ochman and Wilson, 1987). The living descendants of aphids have

maternally inherited endosymbiotic proteobacteria that have radiated synchronously with their host, allowing a calculation of the temporal scale of eubacterial evolution (Moran et al., 1993). By calibrating the endosymbiotic bacterial tree against the fossil aphid record, Moran and her collaborators estimate constant rates of base substitution of 1 to 2% per 50 million years.

The above calculations suggest that the rates of eubacterial 16S rRNA substitution are constant (Ochman and Wilson, 1987; Moran et al., 1993), but other observations indicate that DNA substitution rates change at varying porportions in different organisms and in different times. Thus, calibration of the bacterial clock can be used to calculate the time of divergence of recent prokaryotes like the enterobacteria, for instance, but to extrapolate it back into the early Precambrian epochs, or to attempt to use it to date the origin of life is probably preposterous. Uncorrected extrapolations backward in time suggest that families of different ancient proteins, such as the EF-Tu/EF-G and the actin/heat shock pairs, diverged at unrealistically high values of 10 billion years ago or more, i.e., before the Earth itself was formed (Doolittle, 1992). The limited coding abilities of early RNA genomes and the absence of proof-reading refinements, combined with the relaxation of natural selection following gene duplication, may have been involved in the acceleration of the molecular clock of these genes. Accelerated amino acid substitution rates probably had important evolutionary consequences. Gene conversion mechanisms decline as duplicated genes accumulate point mutations (Walsh, 1987) and, in general, homologous recombination is hindered by sequence divergence (Radman, 1989). Rapid rates of base-pair substitution especially exacerbated by gene duplication and differentiation may have not only limited the effciency of homogenizing mechanisms, but may have favoured rapid evolution of Archean prokaryotes. This phenomenon may be underlie the explosive metabolic evolution that took place soon after the emergence of life (Lazcano and Miller, 1994).

The recognition that major biological innovations may correlate with environmental changes has led to several integrative efforts to compare the patterns of Precambrian biological and geochemical evolution (Knoll, 1992; Margulis, 1993). Although still sketchy, the recognition of what appears to be a pattern of episodic molecular phylogenies of protists is consistent with the rapid increases in eukaryotic microbial diversity and atmospheric oxygen concentrations as inferred from the geological record (Knoll, 1992).

Prokaryotic evolution may have also followed a discontinous pattern. It is possible to distinguish three major episodes of accelerated bacterial diversification associated with (a) the rapid colonization of the early Archean Earth niches; (b) the establishment of an oxidizing environment; and (c) and the evolutionary emergence of plants and animals (Knoll and Lipps, 1993). The most intriguing of these three episodes is the oldest one, which must have been preceded by a period of intense evolutionary experimentation that after a relatively short period of time led to the establishment of the ubiquitous features of mdoern cells (i.e., genome replication, ribosome-mediated protein biosynthesis, energy-generating processes, basic metabolic enzymes, and biosynthetic pathways).

After what appears to be an explosive metabolic evolution that took place soon after the beginning of life, basic genetic processes and major molecular traits appear to have persisted essentially unchanged for more than three billions of years, and represent a prodigious case of evolutionary conservatism (Lazcano and Miller, 1994). The mechanisms involved in generating this large set of traits were extremely succesful. Following the development of oxygen-releasing photosynthesis by the ancestors of cyanobacteria, no new essential biosynthetic traits have appeared. Later events in the evolution of the biosphere involving the emergence of new metabolic pathways (e.g., polyphenols, alkaloids, steroids, lignin, cellulose, etc.) are products of multicellularity and recombination representing only fine tunings and relatively minor additions. Recognition of this pattern raises the issue of the nature of the mechanisms responsible for the lengthy periods of evolutionary stasis during which the emergence of metabolic novelties in different prokaryotic lineages has been strongly limited.

6. FINAL REMARKS

Although in the past few years the relationship between molecular biology and microbial phylogenetics has been embittered by frequent clashes and antagonism, the development of rapidly growing sequence databanks has provided a unique view of the evolution of bacterial and eukaryotic microorganisms, and has opened new perspectives in several major fields of life sciences. Molecular evolution was originally the outcome of the wedding of molecular biology with neodarwinian theory, but it has been rapidly transformed into a field of scientific enquiry in its own right. However, its full development requires not only the development of less-expensive, more rapid macromolecular

sequencing techniques and more powerful computer algorithms for constructing phylogenetic trees, but also the awareness of its non-stated assumptions and more precise definitions of its conceptual framework.

As summarized by Patterson (1988), the theoretical foundations of molecular cladistics have been based on a number of central concepts, most of which were inherited from older disciplines, such as physiology, anatomy, and neodarwinism. Homology, which is one of the key concepts in evolutionary theory, was originally used by Wolfgang Goethe, Ettiene Geoffroy Saint-Hilaire, Richard Owen, and others, to describe structural resemblance to an archetype (Donoghue, 1992). In recent years it has not only been repeatedly confused with sequence similarity (Reeck et al., 1988), but is also used to describe a wider range of possible evolutionary relationships that include species- or gene-phylogeny. In fact, some classes of homology that describe phenomena at the molecular genetic level may have no exact equivalent in orthodox evolutionary analysis of morphological traits. One such case is paralogy, a term coined by Fitch (1970) to describe the diversification of genes following duplication events. Although some aspects of paralogy may be compared to serial homology (which is the phenomenon underlying the appearance of repeated structures in the ontogeny of some metazoa), the former corresponds to duplication events that have occured along the phylogenetic history of a sequence, and not ontogenetically, i. e., as developmental phenomena of an individual plant or animal (Patterson, 1988).

Since paralogy provides evidence of gene duplication but not of speciation events, it is the basis for infering evolutionary relationships among genes, not among species. Recognition of this distinction has led to repeated recommendations on the avoidance of paralogous sequences in phylogenetic analysis (Miyamoto and Cracraft, 1991). However, the use of paralogous duplicates in outgroup analysis for determining the evolutionary polarity of character states in universal phylogenies (Gogarten et al., 1989; Iwabe et al., 1989), has rekindled keen theoretical interest in their advantageous properties. Their use, however, does pose some risks. The naive assumption that only one paralogous duplication has taken place in the set of sequences under consideration may lead to incorrect topologies (Forterre et al., 1993). Indeed, the incorporation of genes that are the result of unrecognized multiple paralogous events in a tree algorithms may be even more insidious than the problems derived by convergent evolution and lateral gene transfer. The latter phenomena are much more easily identified at the molecular level.

The recognition that paralogous duplicates expand a monophyletic group of sequences raises a number of issues not encountered in classical evolutionary analysis. From a (classical) cladistic point of view, a character state that is found only in the outgroups is primitive (Stevens, 1980). Nonetheless, in molecular phylogenetic analysis this may not be always the case. Such rule would hold if multiple paralogous duplications have taken place, and if one (or several) of the older sequences is used as an outgroup for an unrooted tree of younger sequences. This would be the case, for instance, if a myoglobin sequence is used to root alpha (or beta) haemoglobin trees. However, this rule would not hold if an alpha haemoglobin sequence (or a set of them) is used as an outgroup for its the beta haemoglobin tree, or viceversa.

The same is true, of course, with universal phylogenetic trees derived from elongation factors (Iwabe et al., 1989). In this case neither set is older than its homologue. In this case the reconstruction of ancestral character states from dichotomously varying paralogous genes does not comes from the analysis of the outgroup, but may be inferred from the realization that the root of the tree must have been preceded by an even older, more primitive condition in which only one copy of the gene existed prior to the paralogous duplication. Recognition of this fact is likely to play a central role in future understanding of enzyme evolution during the Archean. Although it is true that the raw material for molecular cladistics analysis is restricted to sequences derived from living organisms (or from fossil samples from which ancient preserved DNA can be retrieved) and cannot be applied to extinct groups of organisms, the construction of trees derived from archaic paralogous sequences may allow us to infer evolution prior to the earliest detectable nodes.

The flourishing of molecular techniques has led into a proliferation of sequences of isolated molecular constituents of living organisms, a storehouse of data that has already provided considerable insights into the phylogeny and the diversity of microbes. But because of its very nature, molecular cladistics separates clusters of adaptative characters into a nested hierarchical set which is expected to reflect the temporal sequence of their evolutionary acquisition. However fruitful, such approach has all the demerits of a reductionist one-trait approach to biological evolution chastised in early literature as "partial phylogeny", and since the birth of molecular phylogeny has rarely been used to attempt a truly integrative analysis of complete character complexes.

Such limitation may be overcomed in several ways, some of which are part of intellectual traditions deeply rooted in comparative biology. As Georges Cuvier contended in his 1805 *Lectures in Comparative Anatomy*, the appearance of the whole skeleton can be deduced up to a certain point by examination of a single bone. The success that Cuvier had in such anatomical reconstructions is legendary, and was based not only in his unsurpassed knowledge and intuition, but also on what he termed the "correlation of parts", i. e., the full recognition of a functional coordination of the parts of the body of a given animal (Young, 1992). Such correlation of parts is not restricted to bones and muscles; at subcellular levels, it underlies the functional coordination among the molecular components of multigenic traits such as metabolic pathways and protein biosynthesis. As shown by the intimate relationship between the biosyntheses of valine and isoleucine, their triplet assignments, and the phylogenetic proximity of their aminoacyl-tRNA synthetases, inquiries on the early evolution of the genetic code and other basic features of living systems should be understood not only by determining the molecular phylogenies of some of their isolated components or by mathetical discussions spiced with a distinct Pythagorean flavor, but with the integrative analysis of character complexes.

But for all its foibles, the relationship between molecular biology and evolutionary theory has opened new, unsuspected avenues of intellectual exploration. Never before has such a wealth of methodological approaches and empirical data been available to the students of life's phenomena. In part because of this prosperity, systematics and evolutionary biology, two of the most broadly oriented fields of life sciences, are now in a state of intellectual agitation. The symptoms are manifold; it is possible that the traditional species concept may not apply to prokaryotes, time-cherished concepts like that of the existence of kingdoms are under fire, the origin and taxonomic position of genetic mobile elements is unknown. There is an increased awareness that the understanding of the processes underlying the generation of evolutionary novelties and the origin of ontogenic patterns cannot be restricted by classical neodarwinian explanations. We are living in the midst of hectic times in which epoch-making debates are reshaping the future of the life sciences, and the development of a more integrated molecular biology may be a never-ending story. It is said that to wish someone to live in an interesting time is one of the most terrible of all Chinese curses. Whatever the outcome of current discussions and

debates, for biology the putative Oriental curse may turn out to be nothing less than an intellectual blessing.

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Point Counter Point

Polyphyletic Gene Losses Can Bias Backtrack Characterizations of the Cenancestor

Mushegian and Koonin (1996) have recently published the results of a detailed comparison of the complete genomes of *Haemophilus influenzae* and *Mycoplasma genitalium* in conjunction with the fragmentary data from other organisms available as of March 1996. Once parasite-specific sequences were discarded, the final outcome was an inventory of 256 genes that may resemble, not only the genetic complement of the ancestor of Gram-positive and Gram-negative bacteria, but probably also the amount of DNA required today to sustain a minimal cell. Since most of these sequences have eukaryotic and/or archaeal homologs, Mushegian and Koonin discuss how this figure may be reduced to describe the genome of the last common ancestor (LCA) of the Bacteria, Archaea, and Eucarya, that is, the cenancestor, and suggest how insights on even earlier stages of evolution can be achieved. Given the rapid pace at which more and more cellular genomes are being completely mapped and sequenced, the assumptions and strategies used in such approaches merit considerable attention. As argued here, important pitfalls can be avoided if the polyphyletic gene losses that have taken place in widely separated lineages are properly acknowledged.

The Cenancestor Probably Had a DNA Genome

The backtrack methodology proposed by Mushegian and Koonin (1996) is quite straightforward, and partly based on the idea that genes that are not found in both bacteria and eucarya, or in bacteria and archaea, were probably absent from the cenancestor. The nonstated assumption is that the archaea and eucarya are sister groups, an evolutionary relationship supported by an increasingly larger amount of molecular data. However, such an approach can inadvertently miss nuclear-encoded genes which may have been part of the LCA but lost independently in both the bacterial and archeal domains, or not present in the prokaryotic genomes of a given data set. For instance, the absence in their sample of eukaryotic or archaeal homologs of several key proteins involved in DNA replication led Mushegian and Koonin to speculate that the cenancestor may have had an RNA genome. Several objections can be raised against this conclusion: (1) Sequence similarities shared by many ancient, large proteins found in all three domains suggest that considerable fidelity already existed in the operative genetic system of their common ancestor, but such fidelity is unlikely to be found in RNA-based genetic systems. (2) Sequence analysis and biochemical characterization of a ribonucleotide reductase from the archaeon *Pyrococcus furiosus* has shown that this enzyme shares considerable similarities with both its eubacterial and eukaryotic counterparts (Riera et al. 1997). (3) As underlined by Mushegian and Koonin (1996), their analysis was performed before any complete archaeal or eucaryal genomes became available in the public data bases, and should thus be considered preliminary. Indeed, release of the entire *Methanococcus jannaschii* genome has allowed the identification of one archaeal DNA polymerase exhibiting sequence similarity and three conserved motifs with the eubacterial DNA polymerase II, and with the eukaryotic α, γ, and ε polymerases (Bult et al. 1996). Taken together, these results suggest that DNA genomes and polymerases with proofreading and synthesizing functions evolved prior to the divergence of the three primary kingdoms.

To Salvage or Not to Salvage

Until a more complete data set is available, backtrack inferences on the nature of the cenancestor should be considered as preliminary and perhaps biased by the reduced genomic content of parasites, many of which have undergone multiple secondary losses. For instance, the de novo purine nucleotide biosynthesis is probably one of the oldest metabolic pathways, but it is also one of the

most easily lost by a wide range of obligate symbionts and parasites. Failure to recognize such polyphyletic streamlining processes, which have taken place in *H. influenzae* and at an even greater degree in *M. genitalium,* can lead to some misunderstanding. It would be tempting, for instance, to interpret the absence of purine biosynthesis in the minimal set defined by Mushegian and Koonin (1996) as evidence that the growth and reproduction of the first life-forms depended on the heterotrophic uptake of nucleotides present in the primitive soup (see, for instance, Pennisi 1996). However, such conclusions would be at odds with the problems associated with the chemical synthesis and accumulation under primitive conditions not only of ribose, but also of purine and pyrimidine ribosides, which suggest that none of them are truly prebiotic compounds (cf. Lazcano and Miller 1996).

The phylogenetic distribution of purine nucleotide salvage enzymes can also lead to some confusion regarding the cenancestor's metabolic capabilities. Based on their data set, Mushegian and Koonin (1996) conclude that their minimal cell had the complete nucleotide salvage pathways for all bases except thymine. Adenine deaminase (ADA), which catalizes the hydrolytic deamination of adenine into hypoxanthine, is absent in both *H. influenzae* and *M. genitalium,* and, therefore, was not included in such inventory. However, since the ADA gene is found in other nonpathogenic Gram-positive and Gram-negative bacteria, it may have been part of the LCA genome. The same is probably true of the GMP reductase *guaC* gene. Since GMP reductase is not found in *H. influenzae, M. genitalium, M. jannaschii,* and *Saccharomyces cerevisiae,* it could be argued that the cenancestor lacked *guaC.* Such conclusion is not supported by the presence of GMP reductase in a group of widely separated species that includes *Escherichia coli, Tritrichomonas foetus, Trypanosoma cruzi, Leishmania mexicana,* and humans (Berens et al. 1995). Even organisms with close phylogenetic affinities can differ in their salvage abilities. Hypoxanthine- and guanine phosphoribosyltransferase activities have been found in cell extracts of the euryarchaeota *Methanococcus voltae* (Bowen et al. 1996), but the corresponding genes appear to be absent in the closely related *M. jannaschii,* where the only recognizable purine phosphoribosyltransferase gene is that of adenine PRTase (Bult et al. 1996).

Molecular Phylogenies Are Not Rooted in the Origin of Life

The pioneering work of Mushegian and Koonin (1996) is an important improvement over previous attempts to characterize the LCA (Lazcano 1995 and references therein), but it can be improved by systematic efforts to identify streamlining processes that have led to polyphyletic gene losses in widely separated species. This may be particularly significant given the choice of model organisms whose entire DNA is being sequenced, some of which have been selected because of their relatively small, compact genomes. It is expected that in few years larger volumes of genomic data reflecting a broader cross-section of biological diversity will become available. This will allow not only more precise descriptions of the gene complements of ancestral states, but also an understanding of the effects of parasitism on genomes and the dynamics of gene losses.

Genome sequencing and analysis is rapidly becoming a key element in our understanding of early biological evolution, but it is difficult to see how its applicability can be extended beyond a threshold that corresponds to a period of evolution in which protein biosynthesis was already in operation. Older stages are not yet amenable to this type of analysis. The first life-forms were probably simpler than any cell now alive, and may have lacked not only familiar traits like protein catalysts, but perhaps even genetic macromolecules with ribose–phosphate backbones (Lazcano and Miller 1996). Given the huge gap in our understanding of the evolutionary transition between the prebiotic synthesis of organic compounds and the cenacestor, the temptation to describe the nature of the very first living systems based solely on molecular cladistics should be carefully avoided.

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IN SEARCH OF THE ANCESTRAL RNA POLYMERASE: AN EXPERIMENTAL APPROACH

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ABSTRACT

The hypothesis that contemporary prokaryotic RNA polymerases were initially RNA-dependent, i.e., were replicases, is strongly supported by the observation that cellular DNA-dependent RNA polymerases can use different types of RNA molecules and ribonucleotides as templates. These RNA molecules include RNA viral genomes, viroids, cellular RNAs (tRNA, mRNA, rRNA) and synthetic homo- and heteropolyribonucleotides. The enzyme-mediated DNA-directed RNA synthesis requires a DNA template, ribonucleoside triphosphates, and Mg++. Based on the fact that the template-specificity of both cellular and viral RNA polymerases is reduced in the presence of Mn++, replicase activity has been observed with eubacterial DNA-dependent RNA polymerase. Here we report some preliminary results of our studies on the RNA-directed synthesis of RNA using E.coli RNA polymerase. Optimal replicase activity is observed at 1 mM Mn++, KCl (ionic strength) lower than 3 mM, and slightly basic conditions (pH 8.0 to 8.5). Attempts to observe replicase activity with Zn++ and no Mn++ have been unsuccessful. RNA-dependent RNA synthesis was found to be resistant to actinomycin-D, and is slightly enhanced at 4mM spermidine.

We have also reported here additional evidence of the evolutionary conservation of polypeptides homologous to the eubacterial RNA pol β' subunit by immunochemical studies that have shown that antibodies for the largest subunit (IIo) of calf thymus DNA-dependent RNA pol II also recognize the E.coli RNA pol β' subunit.

PREBIOLOGICAL SELF ORGANIZATION OF MATTER
C. Ponnamperuma and F.R. Eirich (Eds.)

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INTRODUCTION

It has been recently suggested that the first genetic material may have been a polymer made of flexible, acyclic perhaps prochiral nucleotide analogues of non-enzymatic origin which eventually gave way to cellular RNA genomes (Joyce et al., 1987). The existence of cellular systems whose reproduction was based on the replication of a RNA genome has been discussed by a number of authors (cf. Lazcano, 1986). Accordingly, RNA replication must have been achieved by an RNA-dependent RNA polymerase. It has been suggested that vestiges of this enzyme can still be identified as the eubacterial RNA pol β' subunit and its homologues among archaebacterial, eukaryotic and viral RNA polymerases (Lazcano et al., 1987 a, b). This hypothesis is supported by the direct involvement of eukaryotic and eubacterial DNA-dependent RNA polymerases in RNA-directed RNA syntheses under in vitro conditions (Biebricher and Orgel, 1973) and during viroid replication (Lazcano et al., 1987a, b).

The purpose of this paper is to make a preliminary report of our attempts to develop these ideas using an experimental approach. We present results of our studies on a) the ubiquity of polypeptides homologues to the eubacterial RNA pol β' subunit by showing that polyclonal antibodies directed against the largest subunit of calf thymus DNA-dependent RNA pol II recognize the eubacterial β' polypeptide; and b) based on previous work by Biebricher and Orgel (1973) and others, we have established the optimal physicochemical conditions for achieving an RNA-directed RNA synthesis using the E.coli DNA-dependent RNA polymerase.

MATERIALS AND METHODS

REAGENTS

Tryptone, yeast extract, kanamycin, Tris-HCl, sodium ethyldiaminetetraacetate (Na2EDTA), lysozyme, phenylmethanesulphonyl fluoride (PMSF), acetic acid, methanol, glycine, bovine serum albumin, Triton X-100, Coomassie stain, salmon DNA, poly (A,C) and unlabelled ATP were purchased from Sigma Chemical Company. Disodium hydrogen phosphate 12-hydrate, tetrasodium pyrophosphate 10-hydrate, sodium hydroxide, phenol, chloroform, methanol, acrylamide, silver nitrite, citric acid, magnesium chloride, manganese chloride, zinc chloride and ammonium sulphate were obtained from Merck. Sodium dodecyl sulphate, cellulose (Cellex 410), Bio-Gel A 5m, nitrocellulose sheets, protein A, iodogen and TEMED were obtained from Bio-Rad. Glycerol, polymin-P 50% and proteinase-K were purchased from BRL. 2-mercaptoethanol, glycine and sodium deoxycholate were purchased from BDH Chemicals Co. The Scintillation liquid column was obtained from ICN. Dithiothreitol, calf-thymus DNA, unlabelled CTP, GTP and UTP were obtained from Calbiochem Co.. Spermidine was obtained from Aldrich Co. Ethanol, sodium chloride, hydrogen chloride and isoamyl alcohol were purchased from J. T. Baker S. A. de C. V., and 3H-UTP was obtained from New England Nuclear, 125-I from Amersham, and anti-RNA polymerase from Dr. Michael H. Dahmus.

STRAIN AND GROWTH CONDITIONS

E.coli RNase III- was grown to the late log phase in 10% tryptone, %5 yeast extract, 10% NaCl and 0.25 μg/ml kanamycin, previously filtered by Millex-Ha (millipore) 0.45 filer, at 37°C with continuous stirring; 68 g of cells (wet weight) were harvested by centrifugation at 8500 g during 10 min, in a GSA rotor of a Sorvall 2B centrifuge. They were then frozen and stored at -80°C until needed.

BUFFERS

All the buffers were prepared with double-distilled water. Stock solutions were 2.0 M Tris-HCl (pH 7.9), 0.2 M Na2EDIA (pH 7.9), 4 M NaCl and 0.1 M Dithiothreitol. The buffers were 1) Grinding buffer: 0.05 M Tris-HCl, 5% (v/v) glycerol, 2 mM Na2EDTA, 0.1 mM DTT, 1 mM 2-mercaptoethanol, 0.233 M NaCl, 130 μg/ml, lysozyme and 23 μl/ml phenylmethanosulfonyl fluoride (JPMSF); 2) TGED buffer: 0.01 M Tris-HCl, 5% (v/v) glycerol, 0.1 mM Na2EDTA and 0.1 mM DTT which contained different NaCl concentrations depending on the purification step (Burgess and Jendrisack, 1975); and 3) Dialyzing (storage) buffer: 0.01 M Tris-HCl, 50% (v/v) glycerol, 0.1 mM Na2EDTA and 0.1 mM DTT (NaCl was not added).

DNA-CELLULOSE COLUMN

A DNA-Cellulose column was prepared according to Litman (1968). DNA was from calf-thymus; 500 mg of total DNA were suspended in 10 mM Tris-HCl (pH 7.9) and Na2EDTA 1 mM to obtain a solution of 2 mg/ml. This solution was mixed in previously washed and dried Cellex 410. The mixture was exposed to ultraviolet light (10^5 ergs/mm^2) during 45 minutes to fix the DNA to the cellulose. The resultant paste was then dried and lyophilized, and then resuspended and washed several times in the same buffer before packing it into

a column (Pharmacia). The buffer used for washing the paste was scanned in a DU-7 Beckman spectrophotometer (250-310 nm) to confirm the DNA fixation to the cellulose.

RNA POLYMERASE PURIFICATION

The E.coli RNA polymerase purification (Figure 1) was performed at 4°C following the method described by Burgess and Jendrisack (1975); 68 grams of frozen E.coli (ww) were broken and placed in a waring blender containing 150 ml of grinding buffer, and blended at low speed for 2 minutes. The mixture rested for 20 minutes, and then 2.5 ml of sodium deoxycholate were added blending for 30 sec at low speed, and 30 sec at high speed after 20 min. The mixture was centrifugated at 10 000 g for 45 min and the supernatant was then collected; 10 ml of polymin-P 10% were added to it and stirred for 5 min. The mixture was centrifugated 15 min at 8 500 g. The pellet was drained and resuspended in the blender in 200 ml of TGED + 0.5 M NaCl with low stirring during 5 minutes, and then centrifugated at 8 500 g for 30 min. The supernatant was then collected, and its proteins were precipitated with 0.35 g/ml ammonium sulphate, and centrifugated at 10 000 g for 45 minutes. The drained pellet was resuspended in TGED until its conductivity was equivalent to TGED + 0.15 M NaCl. This mixture was passed through a 40 cm DNA-Cellulose column prepared as described above. The column was equilibrated with TGED + 0.15 M NaCl. The mixture was eluted with a 200 ml linear salt gradient (0.15 M to 1 M NaCl). RNA polymerase assays were made, and the reactions in which activity was detected were pooled and passed through a Bio-Gel A 5 m column (Bio-Rad) equilibrated with TGED + 0.5 M NaCl. RNA polymerase assays were repeated. The fractions with a very high activity were pooled (aliquote A2) and separated from the fractions with moderate activity (aliquote B2). Both aliquotes were dialyzed against a storage buffer for one day and stored at -70°C until needed. Protein determination was made following the procedure described by Bradford (1976). The purity of the enzyme at each step was determined by acrylamide gradient (5 to 17.5%) electrophoresis in the presence of SDS (Laemmli, 1970) treated with the $AgNO_3$-stain technique described by Wray (1981).

RNA POLYMERASE ASSAYS

RNA polymerase activity was measured in 50 µl of reaction mixture. The reaction mixture used for activity determination in each step of RNA polymerase purification was 40 mM Tris-HCl (pH 7.9), 5 mM KCl, 8 mM $MgCl_2$, 0.2 mM dithiothreitol, 0.33 mM CTP, 0.33 mM GTP, 0.33 mM ATP, 70 mM UTP 1 µCi 3H-UTP, 1 µg DNA and 10 µl of fraction sample.

The reaction mixture in the determination of optimal conditions varied according to each experiment and is described in the figure captions. The solutions were incubated at 37°C, and the reaction was stopped with 2 µl of 500 mM Na2EDTA. The mixture was bound to Whatman DE-81, and washed several times with 0.8% tetrasodium pyrophosphate, 10% disodium hydrogen phosphate, 0.1% sodium dodecyl sulphate. Dry filters were treated with scintillation liquid, and counted in a Miniaxi Beta 260 Tricarb 4000 (United Technological Packart) liquid scintillation counter. Incorporation of UMP was estimated from the counts per minute and 3H-UTP incorporation.

ASSAYS WITH SPERMIDINE

To measure the effect of spermidine, different concentrations of this polycation (0.0 mM, 0.5 mM, 2 mM and 4 mM) were prepared in a total volume of 145 µl of 10 mM Tris-HCl (pH 7.0), 1 mM NaCl. Samples of 25 µl at each concentration were incubated with 4 µl of 500 µg/µl salmon DNA. or E. coli total RNA at room temperature for 45 minutes to associate the template to spermidine. The assays were then made under the same conditions as described above.

PROTEIN TRANSFER AND WESTERN BLOTTING

Electrophoretic protein transfer from gradient polyacrylamide gels to nitrocellulose was made according to the method described by Towbin et al. (1979) and modified by Burnette (1981). Additional modifications are described below. Total protein from HeLa cells and E. coli RNA polymerase subunits were separated in acrylamide gradient (5 to 17.5%) gels in the presence of sodium dodecyl sulphate in the buffer system of Laemmli (Laemmli, 1970). Each gel was placed in close contact with a nitrocellulose sheet (anode side) in a Bio-Rad Transfer Chamber containing a transfer buffer made of 25 mM Tris-Base, 20% methanol, 192 mM glycine. The transfer was made in 5.5 hours at 500-980 mA at 6°C with cooling to avoid overheating. Following the method described by Garcia-Carranca et al. (1986), the nitrocellulose was blocked with 10 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 0.1% SDS, 3% bovine serum albumine (BSA) during 15 hours. It was then incubated in 3 mM Tris (pH 7.4), 100 mM NaCl, 0.3% Triton X-100, 0.07% SDS, 0.7% BSA and minor amounts of Anti-RNA polymerases II. The nitrocellulose was washed 5 times with the same buffer without BSA; subsequently, the paper was incubated with the same buffer, with BSA and 125-I-labelled protein A, which was prepared according to Tolan et al. (1980). After 6 washes, the nitrocellulose was dried and kept with a XK-1 Kodak plate for several days to detect labelled proteins. The observation of unlabelled proteins was achieved by staining

the nitrocellulose sheet after exposure by the method of Schaffner and Weissmann (1973) as modified to Coomassie stain by Burnette (1981). Molecular markers were included in the gels.

RESULTS

IMMUNOASSAYS WITH ANTI-RNA POLYMERASE II

In order to study the distribution of polypeptides homologous to the E.coli RNA pol β' subunit, Western blots of its SDS-polyacrylamide gel electrophoretic pattern on nitrocellulose sheets were challenged with polyclonal antibodies directed against the calf-thymus DNA-dependent RNA polymerase IIo subunit. A homology character of these two polypeptides is indicated by the positive reaction shown in Figure 2.

REPLICASE ACTIVITY OF THE EUBACTERIAL RNA POLYMERASE

As shown in Figure 3A, RNA-directed RNA synthesis was observed in the E.coli DNA-dependent RNA polymerase, if no KCl was added, Mn++ was used instead of Mg++, and an RNA template (poly A,C or E.coli total RNA) was employed. The absence of any possibly undetected DNA template was confirmed by running assays in the presence of actinomycin-D (0.04 μg/ml). Results are shown in Figure 3B.

In order to achieve an optimal replicase activity, assays were made using different Mn++ concentrations and ionic strengths (Figure 4A), different pH's (Figure 4B), and spermidine concentrations (Figure 5A). Attempts to substitute Mn++ by Zn++ were unsuccessful (Figure 5B). The replicase activity at optimal Mn++ concentration (1 mM Mn++, 0.0 mM KCl) is inhibited by Zn++ (Figure 8); with no Mn++ some replicase activity is observed at 1 mM Zn++. Optimal physicochemical conditions required by the E.coli RNA polymerase when reading either DNA or RNA (poly A,C or E.coli RNA) are summarized in Table 1.

DISCUSSION AND CONCLUSIONS

Homology between the E.coli RNA poly β' polypeptide and the subunit IIo of calf thymus RNA polymerase II is inferred by the cross-reaction of the antibody against the eukaryotic component with the eubacterial subunit (Figure 2). This result is consistent

with previous observations of similar amino acid sequences between the largest component of animal RNA polymerases and the E.coli RNA pol β' subunit (Biggs et al., 1985; Corden, in Biggs et al., 1985). The eukaryotic RNA pol II subunit IIo is a highly phosphorylated polypeptide (Corden et al., 1985). The positive reaction shown in Figure 2 supports the hypothesis that extensive polypeptide chain phosphorylation and other modifications of the nuclear DNA-dependent RNA polymerases largest subunits are secondary adaptations (Lazcano et al., 1987a) that were selected for after the origin of eukaryotic cells (Margulis, 1981).

It is known that under appropriate conditions (absence of a DNA template, Mn++ instead of Mg++) both the Gram-positive (Fox et al., 1964; Adman and Grossman, 1965; Steck et al., 1968) and the Gram-negative (Gomatos et al., 1964; Nigoyi and Stevens, 1965; Biebricher and Orgel, 1973) eubacterial DNA-dependent RNA polymerases will read RNA templates that include tRNA, rRNA, homo-polyribonucleotides and viral RNA genomes. Our obser-ation that RNA polyerase-mediated RNA synthesis is improved, if poly (A,C) is used as a template (Figure 3A) is in agreement with previous observations that have shown that the enzyme reads RNA templates with little secondary structure more efficiently (Biebricher and Orgel, 1973). This suggests to us that the ancestral cellular RNA genomes (Lazcano et al., 1986) may have had only small double-stranded regions.

The results of our studies on the replicase activity of DNA-dependent RNA polymerases are consistent with previous observations on the changes in the template specificity of cellular and viral nucleic acid polymerases in the presence of Mn++ ions (Biebricher and Orgel, 1973; Goodman et al., 1984). Attempts to observe replicase activity of DNA-dependent RNA polymerases in the presence of Mg++, Co++, Fe++ (Biebricher and Orgel, 1973), or Zn++ (Figure 5B) were unsuccessful.

It is well known that DNA-directed RNA synthesis is strongly inhibited by actinomycin-D, an antibiotic that presumably intercalates into the double-stranded DNA template between the base-paired dinucleotide sequence dG-dC (Polya, 1977). Actinomycin-D is known to inhibit E.coli RNA polymerase-mediated viroid replication (Rohde et al., 1982). This may be due to the double helix-like structure of viroids. Other RNA-dependent RNA syntheses are not affected (Sanger and Knight, 1963). It is likely that in our model system with total E.coli RNA replicase the activity was not inhibited by actinomycin-D because the enzyme is reading preferentially RNA molecules with little secondary structure.

Replicase activity of normally DNA-dependent RNA polymerases has been interpreted as vestigial from before the appearance of double-stranded DNA molecules as carriers of cellular genetic

information (Lazcano, 1986; Lazcano et al., 1987 a,b). Table 1 shows that the most significant factors for achieving replicase activity in a DNA-dependent RNA polymerase are 1) the presence of Mn++ instead of Mg++; and 2) a very low ionic strength. The change in template specificity reported here supports the hypothesis that the evolutionary transition from a RNA genome to the use of double-stranded DNA molecules required only minor modifications of the active site of an ancestral RNA-dependent RNA polymerase (Lazcano et al., 1987 a,b). The optimal physicochemical conditions for a RNA-directed RNA synthesis of the E.coli RNA polymerase, summarized in Table 1, will be used in future attempts to establish the replicase activity of the isolated eubacterial RNA pol β' subunit.

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ADDENDUM

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Table 1. E.coli RNA POLYMERASE-MEDIATED RNA SYNTHESIS

	DNA template (salmon)	RNA template (E.coli)
Mn++	---	1.0 mM
Mg++	8.0 mM	---
Zn++	---	---
KCl	100.0 mM	---
pH	7.9	8.0 - 8.5
spermidine	4.5 mM	4.0 mM

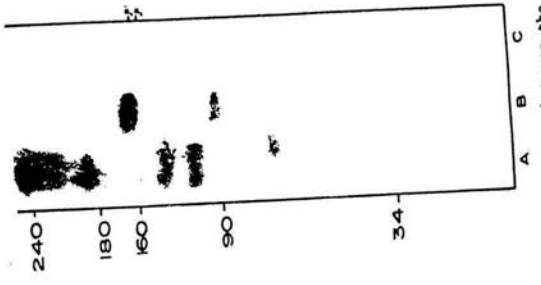

Figure 2. Homology between the subunit IIo of the calf thymus RNA pol II and the E.coli RNA pol β' subunit. Component patterns of the enzymes obtained by SDS-polyacrylamide gel electrophoresis transferred to nitrocellulose filters were challenged with antibody raised against the IIo subunit. Cross-reaction was visualized by 125-I-labelled protein A (see Materials and Methods). Track A, HeLa RNA pol II; Track B, E.coli RNA pol.

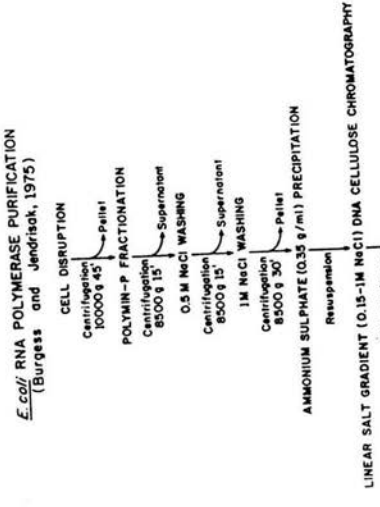

Figure 1. An outline for the purification of E.coli RNA pol (Burgess and Jendrisak, 1975).

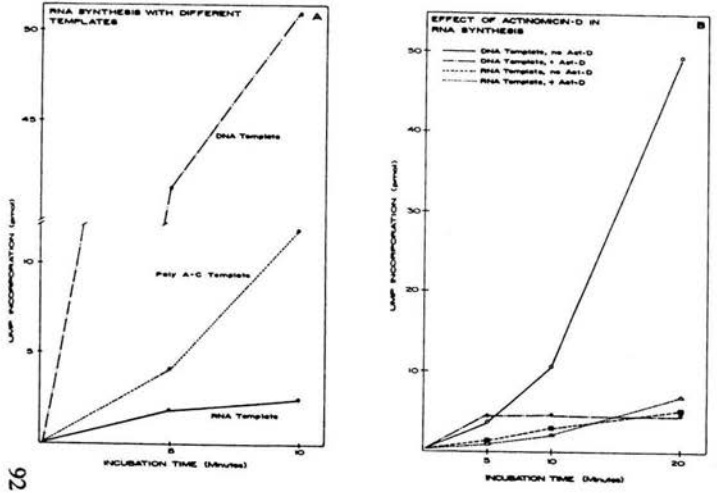

Figure 3

A) E.coli RNA polymerase-mediated RNA synthesis. Activity was measured by UMP incorporation for various intervals, in a 50 μl reaction mixture containing 10 μl of purified enzyme aliquote A2 (see Materials and Methods), 50 mM Tris-HCl, 22 mM $MnCl_2$, 0.2 mM dithiothreitol, 0.22 mM CTP, 0.33 mM GTP, 0.33 mM ATP and 70 μM UTP. The reaction mixture was labelled with 2 μCi 3H-UTP.

B) Effect of actinomycin-D on RNA synthesis. RNA polymerase activity was measured as in 3A. All assays contained 10 μl of purified enzyme (aliquot B2), 50 mM Tris-HCl, 1 mM MnCl2, 0.2 mM DTT, and 1 μg of DNA or RNA. Nucleotides as in 3A.

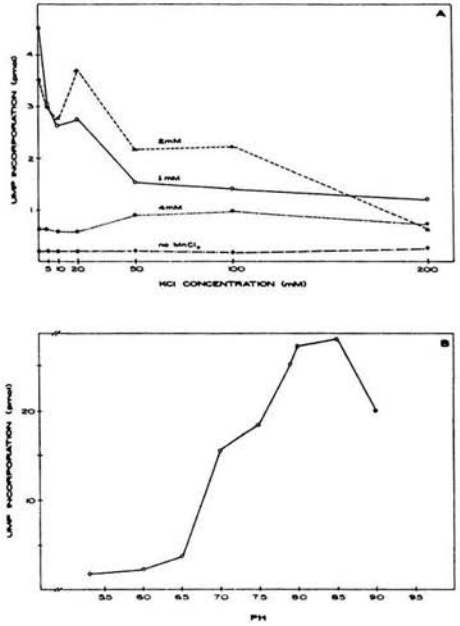

Figure 4

A) Replicase activity of E.coli RNA polymerase with different concentrations of Mn++ and KCl. UMP incorporation (pmol in 50 μl) was measured after 10 minutes in 50 μl of 50 mM Tris-HCl, 0.2 mM DTT, 0.33 mM GTP, 0.33 mM GTP, 0.33 mM ATP, 70 μM UTP, 2 μCi 3H-UTP, with 10 μl of purified RNA polymerase aliquote B2 (see Materials and Methods) and 1 μg of RNA template.

B) Replicase activity of E.coli RNA polymerase at different pH values. The reaction mixture included 1 mM $MnCl_2$, 0.2 mM dithiotreitol, 0.33 mM CTP, 0.33 mM GTP, 0.33 mM ATP, 70 μM UTP, 2 μCi 3H-UTP, 10 μl purified enzyme (aliquote A2), and 40 mM of an acetate or Tris-based buffer with different pH values.

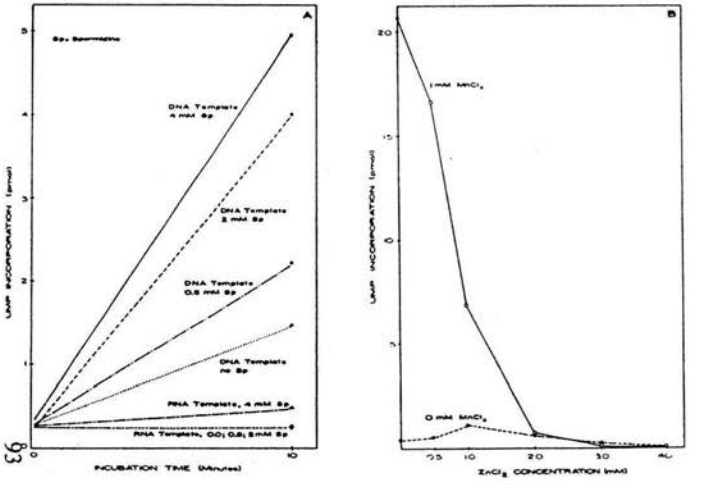

Figure 5

A) Effect of spermidine on RNA polymerase replicase activity. Spermidine was incubated with 2 μg of template (see Materials and Methods), before adding the reaction mixture (50 mM Tris-HCl), 0.2 mM dithiotreitol, 0.33 mM CTP, 0.33 mM GTP, 0.33 mM ATP, 70 μM UTP and 2 μCi 3H-UTP, which contained 10 μl of purified RNA polymerase (aliquote B2).

B) Replicase activity of RNA polymerase in the presence of Zn++. UMP incorporation was measured after 10 minutes of incubation in 50 μl of 50 mM of Tris-HCl, 0.2 mM dithiothreitol, 0.33 mM CTP, 0.33 mM GTP, 0.33 mM ATP and 2 μCi 3H-UTP, with 10 μl of purified enzyme aliquote A2 (see Materials and Methods).

SILVA GONZÁLEZ E. & F.G. MOSQUEIRA PÉREZ SALAZAR. Aspectos funcionales y evolutivos de las aminoacil-tRNA sintetasas. *Rev. Lat-amer. Microbiol.* 33:87-101. (1991)

RESUMEN: Un evento fundamental en la síntesis de las proteínas lo constituye la esterificación del amino ácido a su tRNA correspondiente, catalizado por las aminoacil tRNA-sintetasas.

La importancia de la funcionalidad de esta familia de enzimas en el metabolismo constituye una evidencia de su origen temprano. Como en el caso de muchas otras moléculas que participan en la síntesis de proteínas, la aparición de las aminoacil-tRNA sintetasas se presenta como una cuestión aún no resuelta para explicar el origen de la traducción biológica.

En este artículo se hace una revisión de la información disponible procedente de tres líneas celulares (eubacterias, arqueobacterias y eucariontes) con base en sus propiedades estructurales y catalíticas, con el fin de obtener una mayor comprensión del parentesco evolutivo entre las veinte aminoacil-tRNA sintetasas, tanto para un mismo organismo como entre las diversas líneas celulares.

Entre los resultados más importantes destacan las evidencias en favor del origen monofilético en *Escherichia coli* de la glutamil, glutaminil, y arginil-tRNA sintetasas. Asimismo, se ha podido establecer también el origen monofilético para la leucil, isoleucil, valil, metionil y fenilalanil-tRNA sintetasas de eubacterias, arqueobacterias y eucariontes. Además se ha descubierto un parentesco evolutivo entre las aminoacil-tRNA sintetasas eubacterianas y de organelos (plástidos y mitocondria) así como entre las aminoacil-tRNA sintetasas de origen eucarionte y arqueobacteriano.

SILVA GONZÁLEZ E. & F.G. MOSQUEIRA PÉREZ SALAZAR. Functional and evolutive aspects of aminoacyl-tRNA synthetases. *Rev. Lat-amer. Microbiol.* 33:87-101. (1991).

ABSTRACT: A main event in protein bioshynthesis is the esterification of the correct aminoacid to cognate tRNA catalized by the aminoacyl-tRNA synthetases.

The central role of this family of enzymes in metabolism is an evidence of their ancient origin.

As it is the case in many others molecules involved in protein synthesis, the emergence of the aminoacyl-tRNA synthetases appears to be a problem that is not yet solved in order to understand the origin of the genetic translation.

To obtain a comprehensive view of the evolution of the relationship between each one of the twenty aminoacyl-tRNA synthetases from one organism as well as from different sources (eubacteria, archaebacteria and eukaryotes) we review the information collected from the structural and catalytic properties of these enzyme.

The results allow us to establish the following relationship between aminoacyl-tRNA synthetases. On one side there is a monofiletic origin for glutamyl, glutamynil and argynil-tRNA synthetases from *Escherichia coli* and for valyl, leucyl, metionyl, isoleucyl and phenylalanil-tRNA synthetases from eubacterias, archaebacterias and eukaryotes. On the other side there is an evolutionary relationship between aminoacyl-tRNA synthetases of eubacteria and organelles (plastids and mitochondria) and among eukaryotes and archaebacteria.

Aspectos funcionales y evolutivos de las aminoacil-tRNA sintetasas

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INTRODUCCIÓN

Las aminoacil-tRNA sintetasas, clasificadas dentro del grupo de las ligasas, son un conjunto de enzimas cuya función es la de catalizar la aminoacilación de los tRNAs durante el proceso de traducción biológica.

Por su amplia distribución y función imprescindible en la célula, el estudio y utilización de las aminoacil-tRNA sintetasas como marcadores filogenéticos de los organismos podría permitirnos comprender mejor la interacción que se ha dado entre proteínas y ácidos nucleicos a través de la evolución celular.

Este artículo tiene por objeto dar una visión general de lo que actualmente se conoce sobre las aminoacil-tRNA sintetasas. Sus propiedades generales serán interpretadas con un enfoque evolutivo a fin de intentar dilucidar algunos aspectos de su pasado biológico. Inicialmente se describe la participación de las aminoacil-tRNA sintetasas y su importancia funcional en los procesos de traducción en los organismos contemporáneos.

En la sección II se analizan sus características estructurales, mismas que servirán como criterio para proponer su diversificación temprana, lo cual podrá corroborarse en la sección III con base en las homologías reportadas en estudios de secuenciación en diversos grupos de organismos.

En consideración a la teoría endosimbiótica del origen de las células eucariontes se argumenta en la sección IV sobre la similitud entre las aminoacil-tRNA sintetasas de procariontes con las de algunos organelos de procedencia eucarionte (plástidos y mitocondrias).

En la sección V se discute la utilidad del método experimental de aminoacilación heteróloga como una herramienta de apoyo para estable-

cer el grado de similitud tanto de enzimas de distintos reinos (arqueobacterias, eubacterias y eucariontes) como de uno de sus sustratos (los tRNAs).

En las secciones VI y VII se revisan brevemente los mecanismos de corrección, la participación e importancia evolutiva de los tRNAs y los cationes divalentes en las aminoacil-tRNA sintetasas. En la sección VIII se describe la organización estructural de dichas enzimas de procedencia animal, las cuales conforman un complejo supramolecular constituido de siete a nueve aminoacil-tRNA sintetasas. Por otra parte, propiedades como la homología, naturaleza monomérica, así como requerimientos particulares de catálisis en la glutamil, glutaminil y arginil-tRNA sintetasas de *E. coli* pueden interpretarse como posibles indicadores de un origen monofilético común para dichas enzimas. Asimismo en la sección IX se habla sobre el origen monofilético de la leucil, isoleucil, valil, metionil y fenilalanil-tRNA sintetasas de eubacterias, arqueobacterias y eucariontes.

Finalmente en las secciones VIII y IX se comenta sobre los resultados experimentales con base en técnicas de aminoacilación heteróloga, inmunoensayos y corrección cinética. Dichos ensayos permiten concluir, sorpresivamente, que las aminoacil-tRNA sintetasas arqueobacterianas son en muchas de sus características más parecidas a las de organismos eucariontes que a las de procariontes.

AMINOACIL-tRNA SINTETASAS

I. MOLÉCULAS CODIFICADORAS

En todos los organismos, la manifestación del código genético se realiza por medio de la traducción de los ácidos nucleicos a proteínas. Durante este proceso participan cerca de trescientas macromoléculas[127], entre ellas destacan las que constituyen a los ribosomas, los tRNAs, mRNAs, las aminoacil-tRNA sintetasas y muchas otras. La importancia del estudio de las aminoacil-tRNA sintetasas radica en su función específica que consiste en catalizar la activación del grupo carboxilo del aminoácido por medio del ATP, así como la de mediar en una segunda reacción el enlace covalente de dicho aminoácido con su respectivo tRNA.

El mecanismo de reacción al cual se apega la mayor parte de las aminoacil-tRNA sintetasas estudiadas hasta el momento, está representado con la siguiente ecuación:

$$E + aa + ATP \overset{MgCl_2}{\rightleftarrows} E\bullet AMP\text{-}aa + PPi \quad (I)$$

$$E\bullet AMP\text{-}aa + tRNA \rightleftarrows E + tRNA\text{-}aa + AMP$$

Donde E representa a cualquiera de las veinte aminoacil-tRNA sintetasas con su respectivo aminoácido (aa) y tRNA.

La participación de dichas enzimas resulta esencial, ya que su ausencia en la célula originaría una asignación inespecífica entre aminoácidos y tRNAs, y en consecuencia una síntesis aleatoria de proteínas. Resulta entonces indudable que la transmisión de la información desde los albores de la vida se ha mantenido gracias a la presencia de este grupo de enzimas.

Por esta función codificadora, a las aminoacil-tRNA sintetasas se les ha denominado CODASAS[58], término que adoptaremos en el presente artículo.

II. CARACTERÍSTICAS ESTRUCTURALES DE LAS CODASAS

La primera purificación de una codasa se realizó hace treinta y tres años[49]. A la fecha se ha determinado el peso molecular de no menos de trescientas codasas a partir de diversas fuentes celulares.

La relación en peso molecular que guardan las codasas procariontes con respecto a las animales (anfibios, reptiles, aves y mamíferos) es prácticamente la misma, a diferencia de que estas últimas se presentan agrupadas entre sí configurando verdaderas estructuras supramoleculares.[106]

Las codasas son enzimas con estructura cuaternaria y se les conoce como: $\alpha, \alpha_2, \alpha_4, \alpha\beta, \alpha_2\beta_2$ de acuerdo con el número y característica de la(s) subunidad(es) proteínica(s) que la(s) conforman[116,121].

Las de tipo α son aquellas constituidas únicamente de un monómero proteínico con secuencias internas de duplicación. Las α_2 están conformadas por dos subunidades alfa similares en peso molecular, aparentemente sin secuencias internas de duplicación en cada una de ellas y sin relación con las α inicialmente citadas. Las mismas consideraciones resultan válidas para las α_4. Las $\alpha\beta$ son enzimas cuyos monómeros α y β difieren totalmente entre sí (y de todas las α anteriores) y en donde cada una de ellas presenta secuencias internas de duplicación. El tetrámetro $\alpha_2\beta_2$ lo conforman dos subunidades α_1 iguales unidas a otras dos subunidades β_1 igua-

les pero diferentes de α_1 (ambas subunidades diferentes de α, α_2, α_4 y $\alpha\beta$).[62,106,116,126]

Es importante indicar que dicha clasificación no toma en consideración el grado de similitud de secuencias entre codasas, sino únicamente su naturaleza estructural.

El dato más ampliamente documentado del estudio estructural para las codasas lo encontramos en la fenilalanil codasa. A excepción de la eubacteria *Glicine max* y del protista *Cyanophora paradoxa* así como en organelos de *Euglena gracilis*, las fenilalanil codasas restantes reportadas presentan una estructura $\alpha_2\beta_2$ invariable, por lo que no resulta difícil pensar en la existencia, en dichas codasas, de una secuencia proteínica altamente conservada en la evolución (véase la Tabla I). Desafortunadamente, sólo se conoce la secuencia completa de la fenilalanil-tRNA sintetasa de *Escherichia coli*[107,76].

Es importante señalar que al igual que la fenilalanil-codasa, las diez y nueve restantes conservan su estructura cuaternaria aún en los diferentes reinos (Tabla I) lo que lleva a pensar que seguramente su diversificación estructural se dio en un período temprano de la evolución biológica, donde una vez favorecida por su funcionalidad se han conservado hasta nuestros días.

De las purificaciones logradas resulta difícil establecer una subunidad o estructura común conservada que nos pueda describir la divergencia evolutiva de las codasas; como se ha podido hacer en el caso de otras biomoléculas como las RNA polimerasas.[133] Esto se debe a que el número de pesos moleculares determinado de cada una de las subunidades de las veinte codasas son escasos, así como también lo son las secuencias mismas de las codasas. A pesar de estas limitaciones, sí es posible (en algunos casos) inferir con base en la similitud de secuencias publicadas los fenómenos de duplicación génica que pudieran establecer la naturaleza de su diversificación.

De la comparación de codasas de gran peso molecular, ya sea de cadenas únicas o constituidas por varias subunidades y que se encuentran conformadas además de secuencias aminoacídicas repetidas, es posible inferir un proceso de duplicación y fusión de genes así como también establecer una secuencia ancestral común partiendo de las secuencias proteínicas conocidas.[14,52,60,62,87, 127]

III. HOMOLOGÍA DE SECUENCIAS EN CODASAS

El análisis de las secuencias de aminoácidos (en su mayoría de organismos procariontes) ha permitido establecer el grado o índice de similitud en algunas de ellas.[28,43,107]

La secuenciación de la triptofanil y tirosil-codasas de *Bacillus stearothermophilus* (monómeros de 70 y 80 kDa respectivamente) evidencian homología para sitios con cisteína.[3,61] Estudios de la estructura tridimensional por difracción de rayos x señalan que esta última, a su vez, presenta una notable semejanza estructural a nivel de dominios y de residuos de aminoácidos con la metionil-codasa de *E. coli*[45,48] y su homología con la tirosil-codasa de *Bacillus caldotenax* es tal que su secuencia difiere únicamente en cuatro aminoácidos.[50]

El índice de similitud reportado por Ohnishi (1986) para las triptofanil, glicil, y alanil-codasas en *E. coli*, lo lleva a proponer que dichas codasas tienen su origen a partir de un gen procedente de una codasa ancestral.[83]

Entre las codasas con mayor índice de similitud (diferentes aminoácidos como sustrato) se encuentra una secuencia exclusiva de once aminoácidos consecutivos localizados en la región terminal de isoleucil y metionil-codasas de *E. coli*.[107] La misma secuencia terminal se localiza en la tirosil-codasa de las eubacterias *E. coli, B. stearothermophilus* y *B. caldotenax*.

Asimismo, está descrito que el grado de similitud de esta misma codasa en comparación con la de mitocondria proveniente de *Sacaromyces cerevisiae* es menor,[107] lo cual no es sorprendente si recordamos que las mitocondrias carecen de mecanismos propios de reparación del DNA.[127]

Análogamente existen evidencias de que esta región de once aminoácidos también altamente conservada en la glutaminil, glutamil, valil, leucil, arginil, triptofanil y metionil-codasas de *E. coli*[108] incluye un tetrapéptido His-Ile-Gli-His (HIGH) como sitio de unión al ATP.[107] Las codasas con dicho segmento presentan además un segundo polipéptido (fuera de la región de once aminoácidos) con la secuencia Lis-Met-Ser-Lis-Ser (KMSKS) cuya función se piensa está vinculada al sitio de unión de la codasa con el brazo aceptor del tRNA.[2,28.107] A las codasas con los grupos HIGH y KMSKS se les conoce como codasas de la clase I.[25]

Recientemente Regan y col. (1987) realizaron estudios sobre los probables sitios aminoacídicos de contacto de las codasas con sus sustratos. Ellos utilizaron clonas del gen previamente caracterizado para la alanil-codasa (tetrámero α_4) en *E. coli* y pudieron indentificar aproximadamente el sitio de la enzima que entra en contacto con el tRNA. Sus datos contribuyen a reducir el vacío de conocimientos en esta clase

de interacciones, puesto que, de las nueve codasas arriba citadas, a la fecha sólo se conoce el patrón de difracción cristalográfica de alta resolución del complejo enzima sustrato(s) de la glutaminil-codasa (ATP y $tRNA^{Gln}$) de *E. coli* así como el de la tirosil y metionil-codasas (ATP y aminoácido) de *B. sterotermophilus*.[103]

Por otra parte, los estudios de secuenciación en Seril, Treonil, Prolil, Histidil, Aspargil, Asparginil y Lisil-codasas han establecido la ausencia de los polipéptidos HIGH y KMSKS característicos de las codasas de la clase I.[43] La presencia de tres polipéptidos conservados (ausentes en las codasas de la clase I) en estas siete enzimas, ha llevado a considerarlas como codasas de la clase II.[2,108]

En continuidad con los análisis de secuenciación, la cantidad de estudios practicados en las codasas nos permite identificar una serie de rasgos característicos para tres de las codasas de la clase I: Los de electroforésis en gel de poliacrilamida, la naturaleza monomérica de la glutamil, glutaminil y arginil-codasas (56 kDa,[55] 69 kDa[30] y 63 kDa[17] de *E. coli*.) con pesos moleculares menores a 100 kDa (que representa aproximadamente una cadena polipeptídica de 830 aminoácidos si se toman 120 Daltones como peso molecular promedio de los veinte aminoácidos) y como es de esperarse con un menor número de secuencias de duplicación con respecto a las de peso molecular mayor que 100 kDa.[106] De la comparación entre la glutamil, glutaminil, y arginil-codasas, la máxima similitud hasta el momento identificada se encuentra en las dos primeras. La estructura tridimensional, así como la similitud en sus secuencias consta de siete segmentos próximos al extremo NH_2 terminal.[12,10]

Aún otra propiedad comun entre estas codasas puede señalarse. Los estudios de cinética enzimática realizados por Kern y col. (1979) mostraron que la glutamil, glutaminil y arginil-codasas, a diferencia de otras codasas reportadas, requieren excepcionalmente de sus tRNAs correspondientes para catalizar la incorporación covalente del aminoácido a su tRNA afín. Es decir, en este caso su cinética se representa de la siguiente manera:

$$\text{(II)}\ E + aa + ATP + tRNA \xrightleftharpoons{MgCl_2} aa\text{-}tRNA + AMP + PPi + E$$

La cual difiere de la que se mostró en (I) para la generalidad de las codasas.

El alto grado de similitud en estas tres codasas para *E. coli* ha llevado a algunos autores a proponer que probablemente surgieron a partir de una secuenica ancestral común y que otros dominios proteínicos se adicionaron posteriormente.[12] En resplado a dicha propuesta Kern y col. (1979) han sugerido con base en las rutas biosintéticas de los aminoácidos, que los genes estructurales de glutaminil y arginil-codasas evolucionaron por mutación y duplicación de un gen ancestral de la glutamil-codasa.

Por otra parte, estudios por espectrometría de masas, complementado por el análisis comparativo computarizado de siete codasas secuenciadas parcialmente en *E. coli* (metionil, isoleucil, tirosil, treonil, glicil, triptofanil y alanil-codasas) revelaron la presencia de pequeños segmentos aminoacídicos conservados,[90] siendo más clara esta característica en las primeras cuatro enzimas.[12]

Por lo anterior resultaría bastante interesante analizar el grado de homología de la glutamil, glutaminil y arginil-codasas mediante el mismo algoritmo matemático y comparar estos datos con los de las codasas arriba citadas.

IV. CODASAS EUCARIONTES

Los análisis disponibles de secuenciación de codasas eucariontes proceden casi exclusivamente de las levaduras. En adición a las veinte codasas núcleo-citoplásmicas, los organelos de los eucariontes (plástidos y mitocondrias) presentan también codasas (que llamaremos isoenzimas codasas), para las que se han identificado desplazamientos cromatográficos, reactividad inmunológica y especificidad por el sustrato (tRNA) distintos a los de su contraparte citoplásmica.[6,97]

Se ha sugerido que tanto las codasas como sus respectivas isoenzimas son de procedencia nucleo-citoplásmica, y que las isoenzimas son transportadas al organelo al ser traducidas.[9] La naturaleza del gen o genes que codifican a las codasas e isoenzimas eucariontes es incierta, con excepción de la leucil- y valil-codasa de *E. gracilis* y la fenilalanil-codasa de *Phaseolus vulgaris*, cuya codificación procede de genes distintos, aunque se ignora si la procedencia de ambos genes (la codasa y su respectiva isoenzima) para cada caso sea estrictamente nuclear. Con respecto a este punto, el testimonio más claro que se tiene hasta el momento dimana de las leucil-codasas de cloroplasto de *P. vulgaris* y de la metionil-codasa de mitocondrias de levadura.

Estudios *in vitro* sostienen que dichos genes son de procedencia nuclear.[26,110]

Considerar estos ejemplos como evidencias para poder inferir una hipótesis que describa el origen de las codasas de mitocondria y cloroplastos resultaría prematuro e inapropiado. De hecho, si nuestro marco de estudio estrictamente eucarionte lo extendemos y se realizan comparaciones con organismos procariontes, observaremos que la similitud estructural y funcional de las codasas de estos últimos con respecto a la de organelos es mayor que con respecto a las codasas núcleo-citoplásmicas de organismos eucariontes.[34] Ello resulta explicable si consideramos el origen de organelos desde el punto de vista de la teoría de endosimbiosis.[74]

Así, indicios del origen procarionte de organelos (plástidos y mitocondria) los encontramos en la fenilalanil-codasa de cloroplastos para *P. vulgaris* (dímero de 78 kDa) la cual es similar al de los cianelos de *C. paradoxa*.[95,96] Dicho eucarionte en simbiosis obligada con una cianobacteria (cianelo) representa un estadio de evolución incipiente a cloroplasto.[74,95]

De la misma manera, en ocasiones la semejanza se extiende al índice de similitud determinado en sus secuencias, como sucede con la tirosil codasa eubacteriana (*E. coli*, *B. stearothermophilus*, *B. caldotenax*) y mitocondrial procedente de *S. cerevisiae*.[94,107] Igualmente, se puede señalar que los mecanismos de corrección cinética reportados para las codasas de cloroplastos se asemeja al de eubacterias.[93]

A la fecha el mapeo génico en mitocondria y cloroplastos no ha revelado la existencia de algún gen propio que condifique para una isoenzima. Sin embargo, con la información que hoy día se tiene sobre la constante movilidad génica entre compartimentos eucariontes, resulta factible pensar que dichos genes han sido transferidos en su totalidad al núcleo celular.

V. AMINOACILACIÓN HETERÓLOGA

Algunas de las técnicas experimentales diseñadas para distinguir las zonas específicas de reconocimiento en las codasas con respecto a sus sustratos afines son: el marcaje isotópico, la aminoacilación de moléculas fragmentadas (tRNAs), la formación de complejos covalentes fotoactivados, el análisis de tRNAs mutantes, la aminoacilación heteróloga, etc.[11,44,106]

Los reportes por aminoacilación heteróloga son los que más información han aportado, por lo que a continuación se hará referencia a ellos.

La capacidad de algunas codasas de aminoacilar *in vitro* tRNAs de diferentes grupos de organismos aún dentro del mismo reino (aminoacilación heterólogoa) ha permitido determinar con base en la especificidad de reconocimiento, el grado de parentesco entre procariontes (eubacterias y arqueobacterias) y eucariontes (protistas, hongos, plantas y animales).

Para una mayor comprensión de las investigaciones realizadas por aminoacilación heteróloga, podemos considerar su estudio en organismos pertenecientes exclusivamente a un sólo reino. Así por ejemplo, la fenilalanil-codasas de la arqueobacteria *Methanosarcina barkeri*, aminoacila además de sus tRNAs^{Phe} específicos, al tRNA^{Phe} de *Sulfolobus acidocaldarius* (ambas arqueobacterias). De manera recíproca el tRNA^{Phe} de *M. barkeri* es aminoacilado por la fenilalanil-codasa de *S. acidocaldarius*.[92]

Por otra parte se han observado diversos grados de compatibilidad por aminoacilación entre organismos de distintos reinos. Por ejemplo, se ha descrito que la fenilalanil-codasa de las arqueobacterias *Methanosarcina* y de *Sulfolobus* aminoacilan en mayor porcentaje al tRNA^{Phe} de levadura que al tRNA^{Phe} de *E. coli*. Resultados similares se obtienen al aminoacilar los tRNAs^{Phe} de *Methanosarcina*, *Sulfolobus* y de *E. coli* con la fenilalanil-codasas de levaduras.[92,100,101]

Asimismo la aminoacilación de los tRNAs de la arqueobacteria *Halobacterium cutirubium* por codasas eubacterianas (*E. coli* y *Rhodobacter sphaeroides*) es menor que para codasas eucariontes (levadura).[63] Como conclusión de los estudios de aminoacilación heteróloga se desprende que las eubacterias, arqueobacterias y eucariontes aminoacilan con mayor especificidad tRNAs afines dentro de sus respectivos reinos, mientras que la aminoacilación heteróloga entre dichos grupos es más extensa entre eucariontes (levaduras) y arqueobacterias que con eubacterias.

Las investigaciones por aminoacilación heteróloga permiten también reforzar la hipótesis del origen eubacteriano de las isoenzimas codasas. Mientras la fenilalalnil-codasa citoplásmica del frijol (*P. vulgaris*) no aminoacila tRNAs eubacterianos ni de plástidos, su isoenzima codasa en cloroplastos reconoce a sus respectivos tRNAs, a los eubacterianos (*E. coli*), pero no a los citoplásmicos.[93]

Los reportes por aminoacilación heteróloga han resultado ser un medio eficaz no tan sólo por el hecho de mostrar cuáles son los sitios aminoacídicos de reconocimiento de la enzima hacia sus sustratos, sino también porque han permitido a los investigadores visualizar aspec-

tos de carácter evolutivo. Así, si las codasas son capaces de reconocer *in vitro* tRNAs de fuentes celulares diversas, se debe no sólo a la estructura terciaria que presentan estos últimos, sino también seguramente a la similitud en la secuencia de bases nucleotídicas que los consituyen.

La construcción de árboles filogenéticos recurriendo a las secuencias conocidas de los tRNAs no ha sido un medio eficaz para hacer inferencias sobre la diversificación biológica de los organismos.[119] No obstante, resulta bastante atrayente la idea de construir una filogenia disponiendo únicamente de la interacción heteróloga establecida *in vitro* entre codasas y tRNAs. De hecho este procedimiento se ha utilizado recientemente. En 1988 Hou y Schimmel al trabajar con tRNAs supresores reportaron que el sólo cambio de un par de bases nucleotídicas (G3:U70) en el brazo aceptor del $tRNA^{Phe}$ de *E. coli* propicia su aminoacilación por la arginil-codasa. Debe indicarse que el $tRNA^{Arg}$ justamente presenta el par característico G3:U70. Por otra parte si se lleva a cabo una modificación de bases para que presenten ese mismo par de bases nucleotídicas los tRNAs correspondientes a la cisteína, fenilalanina y tirosina, estos tRNAs pueden ser también aminoacilados por la arginil-codasa. Tales resultados sugieren la sorprendente proposición de que el par de bases nucelotídicas sirve como zona codificadora, esto es, como punto determinante en el reconocimiento del tRNA por la codasa.[80,120]

Esta observación ha ganado aún más solidez con la demostración de que pequeños segmentos de RNA con sólo siete pares de bases y dispuetos helicoidalmente son aminoacilados por la arginil-codasa. La aminoacilación se lleva acabo exclusivamente cuando el par de bases G3:U70 esté incluido en la molécula.[108]

Se sabe que el sólo cambio de dicho par de bases conservado también en otros reinos, como en el gusano de seda *Bombyx mori* y células humanas, conlleva a su no aminoacilación *in vivo* e *in vitro*.[20] Asimismo, la tirosil-codasa de *E. coli* aminoacila los $tRNAs^{Tyr}$ correspondientes a *B. stearotermophilus*, *B. subtilis*, *B. caldotenax*, *E. coli* y mitocondria de *N. crassa*.[7] Dichos experimentos podrían sugerir un mecanismo semejante de reconocimiento.

Afortunadamente, los reportes encaminados a esta línea de estudios resultan cada día más numerosos,[75] por lo que no es sorprendente que en el futuro se confirme la existencia de lo que ya se ha denominado un segundo código genético.[27,111]

Hasta este momento los ejemplos citados de aminoacilación heteróloga corresponden a experimentos de manipulación genética *in vitro*. En cuanto a la aminoacilación heteróloga *in vivo*, sólo se conocen dos casos y son los correspondientes a *Bacilus megaterium* y *Bacillus subtilis*.[56,65] Ambas eubacterias carecen de la glutaminil codasa.[116] La ausencia de la glutaminil codasa en estas bacterias Gram positivas nos podría llevar a pensar que el aminoácido correspondiente, la glutamina, no es activada y en consecuencia estaría ausente de sus cadenas proteínicas sintetizadas. Sin embargo, no sucede así. Al parecer en estos casos la glutamil codasa aminoacila al $tRNA^{Gln}$ y al $tRNA^{Glu}$. Posteriormente mediante una reacción de transaminación la glu-$tRNA^{Gln}$ se transforma en gln-$tRNA^{Gln}$, mediándose de este modo su activación e incorporación a las proteínas.[129]

Se sabe que la glutamil codasa de *B. subtilis* además de aminoacilar a sus respectivos $tRNA^{Glu}$ y $tRNA^{Gln}$, aminoacila *in vitro* a uno de los dos $tRNA^{Gln}$ isoaceptores de *E. coli*. Sorprendentemente, este último difiere del primero en siete bases nucloetídicas.[66] La diferencia en las secuencias nucleotídicas para $tRNA^{Glu}$ y $tRNA^{Gln}$ en *E. coli* y *B. subtilis* oscila de siete a catorce bases en tRNAs que codifican para el mismo aminoácido y de treinta a treinta y ocho bases para aminoácidos diferentes. De lo anterior se propone que el probable sitio común de reconocimiento de los tRNAs aminoacilados por la glutamil codasa de *B. subtilis* corresponde al par G64:C50.[66]

No se conocen investigaciones de aminoacilación heteróloga en *B. megaterium*, así como tampoco las secuencias de sus $tRNAs^{Glu}$ y $tRNAs^{Gln}$. De ser conocidas, resultaría bastante interesante compararlas con las de *E. coli*, además de dilucidar su comportamiento en experimentos de aminoacilación heteróloga de sus correspondientes codasas.

La razón de que la aminoacilación incorrecta *in vivo* se presente como un evento poco frecuente en un organismo, obedece al hecho de que *in vivo* las codasas presentan diversos mecanismos correctores que aseguran su eficacia funcional. Esto explica porqué el orden de error para que se realice una aminoacilación incorrecta es de 10^{-5} [70].

VI. LOS MECANISMOS CORRECTORES DE CODASAS

La selectividad específica de las codasas por sus sustratos se favorece a lo largo de la catálisis enzimática debido a la presencia de varios mecanismos

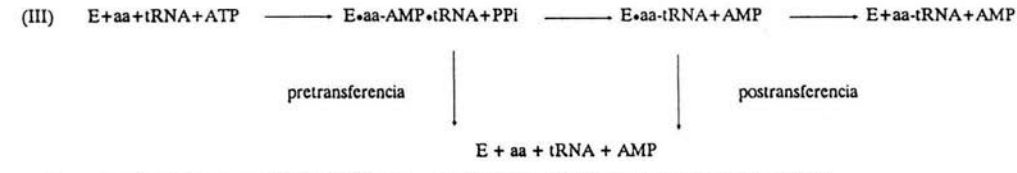
de corrección que restringen la aminoacilación incorrecta de aminoácidos a tRNAs no afines.

De acuerdo al orden y grado de interacción con los sustratos, se han descrito tres mecanismos de corrección: i) corrección cinética, ii) conformacional (pretransferencia) y iii) química (postransferencia).

El mecanismo de corrección cinética, presente al inicio de la reacción, comprende la hidrólisis enzimática del adenilato del aminoácido no específico a la enzima.[70]

Una vez que el tRNA está asociado a una codasa, el mecanismo de corrección puede presentarse antes (pretransferencia) y/o después (postransferencia) de que el aminoácido es transferido al tRNA.[29,48]

Esquemáticamente y de acuerdo a Rauhut, y col. (1986b) se tiene que:



Los mecanismos de corrección enzimática para fenil-codasa (los cuales se describen en su mayoría en presencia de tRNAs) varían de un grupo a otro de organismos.

En el caso de la fenilalanil-codasa de la arqueobacteria *M. barkeri*[92] al igual que la del núcleo-citoplasma de hígado de res,[35,93,33] la vía de corrección más usual *in vitro* con aminoácidos no naturales (p-fluorofenilalanina, ocratoxina B, etc.) es el correspondiente al de postransferencia, mientras que para aminoácidos naturales no afines (metionina, tirosina, leucina, etc.) el de pretransferencia.[96] Por otra parte, es importante destacar que se desconocen reportes de codasas carentes de mecanismos correctores.

Sin duda, la codasa para la fenilalanina es la que está mejor documentada para todos los reinos. Sin excepción su mecanismo corrector presente tanto para aminoácidos naturales como no naturales, es el de postransferencia. Tanto en arqueobacterias, como en el citoplasma de células vegetales y de vertebrados existe el mecanismo adicional de pretransferencia para aminoácidos naturales.

Lamentablemente el estudio de otras codasas no se encuentra tan ampliamente documentado.[47] Sin embargo es de esperarse que la universalidad correctora de postransferencia encontrada en casi todas ellas se deba a su función esencial en la célula, la cual se vió altamente favorecida y conservada durante la evolución.

En contraste con las codasas del núcleo-citoplasma, el mecanismo corrector de postransferencia para cloroplasto de células vegetales y eubacterias está ausente; lo cual puede representar una evidencia sugestiva al considerar la naturaleza eubacteriana de las isoenzimas codasas.

La ventaja evolutiva que representa la presencia del mecanismo corrector de pretransferencia para asegurar la eficacia durante la aminoacilación en células vegetales se percibe mejor al observar sus condiciones fisiológicas propias.

En *Convallaria majalis* y *Mimosa pudica* (planta liliacea y leguminosa respectivamente) y en general, en la mayoría de las plantas, se lleva a cabo la síntesis de un gran número de aminoácidos naturales distintos a los veinte comúnmente identificados en la síntesis de proteínas.[31] Su presencia en la célula vegetal conlleva al establecimiento de competencia del sitio activo de alguna de las codasas específicas para los veinte aminoácidos codificados. Así, durante la diversificación biológica de los organismos, la permanencia de mecanismos de corrección como el de pretransferencia puede explicarse por la eficiencia que dicho mecanismo establece durante la traducción en la síntesis de proteínas.

Con respecto a la manera como se realiza la corrección enzimática de pretransferencia, se ha observado que no necesariamente se requiere que en la enzima el tRNA interaccione con el adenilato del aminoácido para mediar la desactivación del aminoácido erróneamente activado, lo que puede interpretarse como un mecanismo de corrección propio de la enzima y no de los sustratos.[56]

El mecanismo de corrección de postransferencia que origina la desaminoacilación del tRNA erróneamente aminoacilado no resulta tan fácilmente explicable, ya que en el proceso, además de la enzima, entran en juego los sitios activos 2'OH y 3'OH de la ribosa en la adenina del extremo CCA del tRNA.

Las diferentes explicaciones al porqué de la elección en la especificidad inicial de aminoacilación por el sitio 2'OH o 3'OH y de una eventual desaminoacilación en el mecanismo de corrección, no han resultado del todo satisfactorias. Hecht (1980) propone que la selectividad por uno de los sitios obedece a la relación de polaridad que guardan los aminoácidos y el segundo nucleótido del anticódon del tRNA que los codifica. Quizá ello explica en cierta manera que en los tres reinos el $tRNA^{Phe}$ tenga como sitio inicial de aminoacilación la posición 2'OH de la ribosa.

Independientemente de cualquier otra explicación que se dé al respecto, resulta evidente que el tRNA juega un papel preponderante tanto en el mecanismo de aminoacilación como de desaminoacilación. No resultaría sorprendente encontrar evidencias contundentes de la funcionalidad del tRNA en dichos procesos.

Recientemente se ha evidenciado que la molécula de tRNA además de servir como transportador de aminoácidos en la síntesis de proteínas realiza otras funciones,[38,82] entre ellas la de actuar como cebador, como en el caso del $tRNA^{Trp}$ durante la iniciación en la síntesis de DNA catalizada por la enzima reversa transcriptasa.[86] Así mismo, de extractos celulares de la paratiroides se sabe que la $tRNA^{Arg}$ incorpora sus respectivos aminoácidos a secuencias peptídicas en ausencia de ribosomas.[54] Por otra parte una de las rutas de biosíntesis del ácido aminolevulínico, precursor de la porfirina, requiere como sustrato la presencia de $tRNA^{Glu109}$ También se ha podido identificar que la incorporación del aminoácido arginina y glicina en la biosíntesis de pared celular bacteriana es mediada por sus correspondientes tRNAs sin hacer uso del sistema ribosomal.[81,99] Finalmente, se conoce la participación del $tRNA^{His}$ en la expresión de la regulación génica a nivel de cromatina[18] y, como ya se ha mencionado, posiblemente como efector alostérico en el mecanismo corrector de pretransferencia y postransferencia. Seguramente, la mayoría de estas funciones adicionales del tRNA fueron capitales en los albores de la formación de metabolismos bacterianos, al poder realizar ciertas funciones por medio de dichas macromoléculas de naturaleza no proteínica.[36,64]

VII. CATIONES DIVALENTES

Numerosas son las enzimas que requieren en su estructura de iones metálicos para llevar a cabo eficientemente su catálisis.[123] Es plausible suponer que los iones metálicos jugaron un papel preponderante en los inicios del metabolismo bacteriano al poder llevar a cabo de manera incipiente algunas funciones catalíticas vitales en los primeros organismos, como se ha propuesto repetidamente.[15,53,71,84,85] Sin embargo, hacer una sugerencia semejante en cuanto al efecto de los iones metálicos con respecto a la función que realizan las codasas es todavía prematuro.

Los requerimientos catalíticos aún en las distintas codasas de un mismo organismo varían notoriamente en función de sus exigencias metabólicas.[84,116]

Entre los cationes disponibles intracelularmente, el Mg^{2+} resulta ser el más efectivo e indispensable durante todo el proceso de aminoacilación.

No obstante que las codasas no constituyen un grupo estricto de metaloenzimas, la metionil e isoleucil codasas de *E. coli* y *B. stearotermophilus* constituyen las únicas codasas investigadas donde el catión zinc está localizado como parte integral en los protómeros estructuraels.[73,89,113]

En ocasiones la eficiencia catalítica de las codasas depende en gran medida de las concentraciones intracelulares de zinc. Un caso de sumo interés es el de la fenilalanil-codasa de la arqueobacteria *M. barkeri*, la cual para su estimulación *in vitro* requiere de una concentración de zinc de ocho a diez veces mayor que la requerida en la levadura, *E. coli* e hígado de cordero.

Poco se conoce de la participación del zinc en la catálisis de las codasas; no obstante su efecto parece estar vinculado a las funciones regulatorias de moléculas efectoras tales como el del Ap_4A (diadenosin tetrafosfato) en los tres reinos.[88,94,121]

Con respecto a la participación adicional de las codasas en procesos colaterales a la transcripción y traducción, se han referido estudios para la arginil, alanil y treonil codasas. En el primero y segundo caso, su función es la de autorregulación de su síntesis durante la iniciación de la transcripción,[12] mientras que la tercera actúa uniéndose al extremo 5' del mRNA (en una secuencia homóloga a su tRNA) inhibiendo la traducción.[102] Análogamente, otras publicaciones recientes[41] demuestran la participación *in vitro* de leucil-codasa mitocondrial de *S. cerevisiae* y la tirosil-codasa citoplásimica de *N. crassa* en el procesamiento de su pre-mRNA.[41,72]

Estos autores han argumentado que este es un caso evidente de evolución incipiente, puesto que sus observaciones en el procesamiento del mRNA mitocondrial para el caso de la *S. cerevisiae* muestran una gran similitud con la *S. douglasii*.

VIII. LAS CODASAS ANIMALES

Las codasas purificadas de animales (anfibios, ptiles, aves y mamíferos) se presentan en comejos moleculares constituidos de tRNAs, facres de alargamiento, lípidos, proteínas y otros mponentes aún no determinados. En conjunconforman una estructura molecular de aprodamente 10^3 kDa.[22,25,79] Dichos complejos tán descritos para muy variadas fuentes celulas, desde células HeLa hasta oocytos de *Xenos leavis*.[25,79,107]

La composición del complejo que puede dicidarse a través de la actividad de cada uno de s componentes extraídos durante su aislaiento, muestra la presencia *in vitro* de un complejo enzimático[4,13,16,40,91,105,112,118] el cual en casiones llega a constituirse de siete a nueve iferentes tipos de codasas.[21,24,77,107,122,125] Enre ellas la metionil, leucil, lisil e isoleucil-codas, siendo éstas las que se encuentran en mayor bundancia, y seguidas por arginil, glutaminil, lutamil, prolil y aspargil-codasas que se preentan en menor proporción.[13,21,57]

Al explorar las propiedades funcionales por nedio de análisis cinéticos, se ha podido demosrar que las siete codasas constituyentes del comlejo enzimático en hígado de cordero carecen le interdependencia funcional.[78]

La organización *in vitro* de un complejo estructural y el significado fisiológico que cumple son aún desconocidos.[104,107,115]

Se piensa que su existencia *in vivo* en forma de heterocomplejo (codasas diferentes) cumple funciones regulatorias en la síntesis de proteínas.[23,114] Sin embargo dicha aseveración no se ha demostrado experimentalmente.

La identificación de un complejo multienzimático en *E. coli* en el cual una codasa puede estar unida a otras enzimas (no necesariamente otras codasas) constituye la única evidencia de agregados moleculares que albergan codasas procariontes. La organización y constitución de tales complejos difiere a la que presentan los eucariontes.[37]

IX. LAS CODASAS CON MAYORES SIMILITUDES

En general puede decirse que en *E. coli* las codasas arginil, glutamil y glutaminil-codasas, que pertenecen a la clase I, son las que presentan una mayor similitud. Aunque no debe perderse de vista la semejanza observada entre la leucil, valil, fenilalanil e isoleucil-codasas de diversos organismos.

1. Glutamil, Glutaminil y Arginil-codasas.

Entre las evidencias más sobresalientes en cuando a la similitud entre las glutamil, glutaminil y arginil-codasas encontramos, al igual que el resto de las codasas de la clase I, la de sus homologías en estructura monomérica, así como la de sus requerimientos catalíticos. En efecto, en este grupo de codasas se requiere de la presencia del tRNA para que se pueda realizar íntegramente su función catalítica (véase ecuación II). Esta característica las agrupa como únicas en cuanto a su vía particular de catálisis (probablemente primigenia) en donde a falta de una estructura cuaternaria enzimática funcionalmente eficiente, se requiere del tRNA para inducir la activación del aminoácido y su esterificación al tRNA afín[67] apartándose del patrón cinético que se observa en la mayoría de las codasas (véase ecuación I).

Esta interpretación evolutiva resulta atractiva si consideramos que los sitios catalíticos en algunas codasas con estructura dimérica se encuentran parcialmente distribuidos en cada una de las subunidades proteínicas que conforman la estructura cuaternaria de la enzima.[107] Por ejemplo se ha podido comprobar en el caso de la tirosil-codasa de *B. stearothermophilus* que la disociación de su estructura dimérica en sus respectivos monómeros simétricos conlleva a una disminución considerable en la afinidad de activación del aminoácido y su aminoacilación al tRNA correspondiente.[50] Lo anterior puede sugerir una evolución conjunta de las partes monoméricas que constituyen a dicha codasa, las cuales pudieron haber surgido, probablemente, por medio de un proceso de duplicación de genes.

En contraste, la glutamil, glutaminil, arginil-codasas, son enzimas monoméricas, lo cual puede interpretarse como enzimas verdaderamente primigenias con respecto al resto de las codasas, ya que al menos requieren de un menor grado de estructuración para actuar catalíticamente.

La homología en las secuencias de glutamil, glutaminil y arginil-codasas se refleja también en los estudios de secuenciación de sus respectivos tRNAs[66,119] en donde, notoriamente, el sitio inicial común de aminoacilación ocurre en el extremo 2'OH del adenosín (extremo CCA del tRNA). La poca variación estructural para las codasas, y en particular para la glutamil, glutaminil y arginil-codasas nos lleva a pensar que posiblemente una vez que aconteció su diversificación estructural y funcional, ésta se vió favorecida a lo largo de la evolución.

Si al igual que otras enzimas estudiadas como las RNA polimerasas,[68] el grado de complejidad funcional existente en las codasas restantes se con-

sidera como producto de su diversificación por un proceso de duplicación y/o fusión de genes, y además se acepta que una mayor complejidad estructural de la codasa facilita el llevar a cabo mecanismos de control y corrección, cabría esperar encontrar en la glutamil, glutaminil y arginil-codasas un bajo rendimiento en su actividad correctora, lo cual no ha podido todavía verificarse experimentalmente. Finalmente, el estudio detallado de la cinética enzimática para estas tres codasas en los distintos grupos de procariontes podrían servir como base de comparación de posibles rasgos comunes en el mecanismo de corrección cinética de dichas codasas.

2. Valil, leucil, Isoleucil, Metionil y Fenilalanil-codasas

La aminoacilación heteróloga *in vitro* ha sido otra técnica experimental que ha permitido identificar grupos de codasas con características comunes. Las investigaciones de las codasas a través de dicho estudio pueden interpretarse como evidencia adicional del origen común del grupo de codasas constituído por la valil, leucil, isoleucil, metionil y fenilalanil-codasas. Así, por ejemplo, el hecho de que la fenil-codasa de levadura pueda aminoacilar al $tRNA^{Met}$, $tRNA^{Val}$, $tRNA^{Ile}$, $tRNA^{Leu}$ y $tRNA^{Phe}$, todos ellos correspondientes a aminoácidos hidrófobos, permite inferir que esto es posible gracias a la posición común de ciertas bases presentes en dichos tRNAs.[63,94] No obstante que se desconoce experimentalmente la base o conjunto de bases comunes que facilitan el reconocimiento, se ha propuesto la presencia de una base común conocida como base discriminadora, la cual corresponde a la cuarta posición del extremo 3' del tRNA.[19] De acuerdo con esta hipótesis, la presencia de dicha base discriminadora se ha mantenido en la evolución conservando la afinidad química que guardan entre sí este grupo de aminoácidos de naturaleza hidrofóbica con sus respectivos tRNAs. La caracterización de los cuatro tRANs correspondientes a los aminoácidos hidrofóbicos de *E. coli* y levadura revelan algunos rasgos comunes entre ellos, como por ejemplo, la presencia de adenina como base discriminadora y su correspondiente aminoacilación inicial en el extremo 2'OH de la ribosa.[128] Analizando la secuencia de bases de los cuatro tRNAs de estos organismos, se observa que sus respectivos aminoácidos son codificados por uracilo como base central del codón y, finalmente, sus secuencias nucleotídicas presentan un elevado índice de similitud,[119] a partir de las cuales la zona de reconocimiento propuesta en estos tRNA consta de nueve nucleótidos del asa D y cuatro del brazo aceptor. Esta propuesta la han apoyado Vlassov, V. et. al. (1983) quienes proponen además del asa D, al asa variable y al anticodón como zonas de reconocimiento por las codasas.

Así mismo resulta interesante observar que el extremo 3'OH del virus de mosaico del tabaco, como también el del nabo amarillo, sean aminoacilados con valina en presencia de ATP y valil codasa de *E. coli*.[8,47] La homología de estos RNA virales permite comprender, por su simplicidad, el origen de los sitios de reconocimiento primigenio de los tRNAs por las codasas.[69] De los sitios descritos en dichos virus destaca la secuencia CCA común a los tRNAs (indispensable para su aminoacilación) y en donde al igual que en estos últimos la aminoacilación ocurre.[47]

CONCLUSIONES

La información recabada de las codasas no permite todavía identificar una secuencia común ancestral conservada en todas ellas.

Sin embargo, dicha posibilidad no queda descartada y algunas características comunes en glutamil, glutaminil y arginil-codasas, como lo es por ejemplo su estructura monomérica, la homología de secuencias y de sus respectivos sustratos (los tRNAs) cuya participación en la reacción de aminoacilación resulta indispensable, nos permite apoyar la hipótesis de que dichos rasgos, únicos para este grupo de enzimas, constituyen vestigios de su origen monofilético.

Por otra parte, los estudios experimentales por medio de la aminoacilación heteróloga y secuenciación, aunados al carácter apolar de los aminoácidos correspondientes, han permitido agrupar a la valil, leucil, isoleucil, metionil y fenilalanil-codasas como otro grupo de codasas con características muy semejantes que también pudiera sugerir un origen monofilético para dichas codasas.

La relación evolutiva entre estos dos grupos de codasas no se ha analizado hasta el momento.

Las investigaciones que utilizan técnicas por inmunoensayos, aminoacilación heteróloga y mecanismos cinéticos de corrección en eubacterias, eucariontes y arqueobacterias, constituyen una fuerte evidencia del origen eubacteriano de las codasas de organelos (plástidos y mitocondrias). Asimismo, otras evidencias experimentales hacen suponer que existen rasgos comunes entre las codasas eucariontes y arqueobacterianas.

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TABLA I

ESTRUCTURA CUATERNARIA DE CODASAS IDENTIFICADAS PARA ALGUNOS ORGANISMOS

	Leu	Ile	Val	Met	Phe	Ser	Pro	Thr	Ala	Tyr	His	Gln	Asp	Asn	Lis	Glu	Cys	Trp	Arg	Gly
E. coli	α	α	α	α	$\alpha_2\beta_2$	α_2	α_2	α_2	α_2	α_2	α_2	α	α_2	-	α_2	α	-	α_2	α	$\alpha_2\beta_2$
B. stearothermo	α	α	α	α_2	-	α_2	-	α_2	-	α_2	-	-	α_2	α_2	α_2	α	α	α_2	α	-
B. subtilis	α	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. brevis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	$\alpha_2\beta_2$
S. typhimurium	-	-	-	-	$\alpha_2\beta_2$	-	-	-	-	-	α_2	-	-	-	-	-	-	-	-	-
M. barkery	-	-	-	-	$\alpha_2\beta_2$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. smegmatis	-	-	α_4	α_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae	α_2	α	α	-	$\alpha_2\beta_2$	α_2	-	-	α	α_4	-	-	-	-	α_2	-	-	α_2	α	-
S. carlsbergensis	-	-	-	-	-	α_2	-	α_2	-	-	-	-	-	-	-	-	-	-	-	-
levadura de pan	-	-	-	-	-	-	-	α_2	-	α_2	-	-	-	-	-	-	-	-	-	-
N. crassa, cito.	α	-	-	-	$\alpha_2\beta_2$	-	-	-	-	-	-	-	-	-	-	-	-	-	α	-
mito.	α	-	-	-	-	-	-	-	-	-	-	-	α_2	-	-	-	-	-	-	-
levadura, mito.	-	-	-	α_2	$\alpha_2\beta_2$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. utilis	α	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. nidulans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. gracilis, cito.	α	-	α	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
mito.	α	-	α	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
semillas luteus	-	-	α	α_2	$\alpha_2\beta_2$	α_2	-	-	-	-	-	-	-	-	-	-	-	α_4	-	α_2
soya, cito.	-	-	-	-	-	-	-	-	-	α_2	-	-	-	-	-	-	-	-	-	-
mito	-	-	-	-	-	-	-	-	-	α_2	-	-	-	-	-	-	-	-	-	-
L. luteus	-	-	-	-	$\alpha_2\beta_2$	-	-	-	-	α_2	-	-	-	-	-	-	-	-	-	-
semilla de trigo	-	-	-	-	$\alpha_2\beta_2$	-	-	-	-	-	-	-	-	-	-	-	-	-	α_2	-
P. vulgaris, cito.	α_2	-	-	-	$\alpha_2\beta_2$	-	-	-	-	-	-	-	-	-	-	-	-	-	α	α_2
cloro.	-	-	-	-	$\alpha\beta$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
hígado de res	-	-	-	-	$\alpha_2\beta_2$	α_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
hígado de cordero	-	-	-	-	$\alpha_2\beta_2$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	α
glds. mam. de cor.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	α
hígado de rata	-	-	-	-	$\alpha_2\beta_2$	-	-	α_2	-	α_2	-	-	-	-	-	-	α_2	-	-	-
retículo de conejo	-	-	-	-	-	-	-	-	-	-	α_2	-	-	-	α_2	-	-	-	-	-
páncreas de res	-	-	-	-	$\alpha_4\beta_4$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cerebro de res	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	α_2	-	-
páncreas de bovino	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	α_2	-	-
páncreas de cerdo	-	-	-	-	-	-	-	-	-	α_2	-	-	-	-	-	-	-	-	-	-
glds. mam. de vaca	α_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	α_2	-	-
placenta humana	-	-	-	-	$\alpha_2\beta_2$	-	-	-	-	-	-	-	-	-	-	-	-	α_2	-	-
piel humana	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	α_2	-	-

Datos recopilados de las Referencias 49,106 y 117.

El símbolo (-) indica que su estructura cuaternaria no se ha publicado.