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ESTACION EXPERIMENTAL DEL ZAIDIN. GRANADA

ACTIVIDAD CATALASICA DE MUTANTES DE *ESCHERICHIA COLI* Y *STAPHYLOCOCCUS AUREUS* CON DEFICIENCIA RESPIRATORIA

POR
E. MONTOYA

El uso de mutantes de microorganismos con deficiencia respiratoria para la búsqueda de antibióticos anticancerosos (5-6, 8), se basa, de acuerdo con la teoría de Warburg sobre el origen de la célula cancerosa (11-12), en la hipótesis de que dichas mutantes son los equivalentes del cáncer en los microorganismos. Esto ha traído como consecuencia que sea de gran interés el estudio de las citadas mutantes con deficiencia respiratoria, ya que es evidente que la coincidencia o semejanza de sus propiedades bioquímicas con las de las células cancerosas no sólo confirmaría su utilidad como organismos de prueba en la búsqueda de sustancias anticancerosas, sino que, en último término, reforzaría la teoría de Warburg.

Warburg (13) tiene la opinión de que una de las características bioquímicas más interesantes de la célula cancerosa es su baja actividad catalásica.

En relación con ello, Callao y Montoya (2) han demostrado que el contenido en catalasa de las mutantes de *Saccharomyces cerevisiae* con deficiencia respiratoria es mucho menor que el de la raza original y, asimismo, Gause (6) cita una experiencia con mutantes de *Staphylococcus aureus* que indican también una disminución de la actividad catalásica en las mismas.

El presente trabajo tiene por objeto efectuar un estudio sobre la actividad catalásica de mutantes de *Escherichia coli* y *Staphylococcus aureus* con deficiencia respiratoria, para comprobar si los resultados de Callao

y Montoya (2) pueden ser extendidos a las bacterias y completar y confirmar así los datos de Gause (6) sobre *S. aureus*.

MATERIAL Y METODOS

Organismos

Las experiencias se han llevado a cabo con una raza de *E. coli*, la SS-47$_1$, aislada en nuestro laboratorio a partir de una diarrea infantil, y otra de *S. aureus*, la C$_4$, aislada de un forúnculo.

Obtención de las mutantes con deficiencia respiratoria (DR)

E. coli

Las mutantes DR de *E. coli* se obtuvieron por tratamiento de la raza original con luz ultravioleta y uretano.

El proceso con luz ultravioleta se desarrolló de la siguiente manera: suspensiones en solución salina procedentes de un cultivo de veinticuatro horas en agar común y con una densidad aproximada de 5×10^9 células/cm^3, se diseminaron en la superficie de placas de agar común; las placas así inoculadas se sometieron seguidamente a la acción de la luz ultravioleta hasta que se consiguió la muerte del 99-99,9 por ciento de las bacterias inoculadas y fueron incubadas a 37 °C durante una semana, efectuándose observaciones diarias sobre el desarrollo de las colonias. Se seleccionaron como posibles mutantes DR aquellas colonias que mostraron menor desarrollo y cuya aparición fue más tardía.

La obtención de mutantes DR mediante uretano se llevó a cabo, de acuerdo con Gause (4), cultivando la raza original de colibacilo sobre agar común con el 2 por ciento de uretano y seleccionando algunas de las muy escasas colonias que crecieron en este medio.

S. aureus

Las mutantes DR de este germen se obtuvieron mediante tratamiento con luz ultravioleta, siguiendo la misma técnica descrita para *E. coli*. Se seleccionaron las colonias que presentaron un menor desarrollo y un ligero pigmento rosa-anaranjado.

En general, el rendimiento en mutantes DR fue muy escaso, lo que está de acuerdo con los resultados obtenidos por Gause y cols. (7), los cuales encuentran que, en condiciones óptimas, la frecuencia de aparición de mutantes DR es de 1,04-1,07/10^8 células, en el caso de *S. aureus* tratado con luz ultravioleta.

Determinación de la actividad respiratoria

La actividad respiratoria de las razas originales, así como de las obtenidas por los procedimientos descritos, se valoró determinando su Q_{O_2} (µl de O_2, hora/mg de peso seco) por la técnica manométrica de Warburg. Para realizar la determinación se empleó, en el caso de razas de *E. coli,* tampón de fosfatos 0,01M, pH 7, con el 0,5 por ciento de glucosa, y, en el caso de razas de *S. aureus,* un medio con glucosa 0,0555M; bicarbonato sódico 0,0003M, y cloruro sódico 0,15M (4).

En todos los casos se emplearon células lavadas procedentes de un cultivo de veinticuatro horas en agar común. En el caso de las razas obtenidas por tratamiento con uretano, se usó como medio de cultivo para la obtención de la masa microbiana, agar común con el 2 por ciento de uretano.

Efecto del KCN 0,01M sobre la actividad respiratoria

Para comprobar la deficiencia respiratoria de las mutantes aisladas, se estudió el efecto del KCN 0,01M sobre la respiración de las mismas. Se usó la misma técnica y medios que para la determinación de la actividad respiratoria. Para evitar los errores debidos a la fijación de HCN sobre la potasa al 10 por ciento del pocillo central de los frascos manométricos, se utilizó, en sustitución de aquélla, una mezcla de 0,1 cm^3 de KOH 0,002M, y 0,1 cm^3 de KCN 2M (10).

Identificación de las mutantes DR

Se identificaron como mutantes las razas seleccionadas cuyo Q_{O_2} resultó ser un 50-60 por ciento menor que el de las razas originales y cuya actividad respiratoria se redujo solamente un 60 por ciento o menos en presencia de KCN 0,01M.

Determinación de la actividad catalásica

Se efectuó sobre los homogeneizados correspondientes, obtenidos al suspender en 3 cm^3 de tampón de fosfatos 0,01M, pH 7, las células obtenidas de un cultivo de veinticuatro horas en frasco de Roux, previamente desecadas a la acetona y pulverizadas de acuerdo con la técnica de Gunsalus y Umbreit (9).

Se utilizó el método manganimétrico de Bonnichsen y cols. (1), y las determinaciones se efectuaron siempre entre 0-4 °C. Los resultados se expresaron como *Kat. f.* (*), calculada mediante las siguientes ecuaciones:

$$Kat.\ f. = \frac{k}{p}\ cm^3\ g^{-1}\ min^{-1}$$

donde p es el peso en gramos de la muestra empleada en la determinación y k la constante de velocidad de la seudorreacción de primer orden producida por la catalasa:

$$k = \frac{\log.\ (x_o/x)}{t}\ cm^3\ min^{-1}$$

siendo x_0 la concentración inicial de substrato expresada en centímetros cúbicos de $KMnO_4$ 0,01N y x la concentración de substrato, expresada en los mismos términos, a los t minutos.

RESULTADOS

Mutantes DR obtenidas

Mediante las técnicas expuestas fueron obtenidas e identificadas como tales dos mutantes DR de *E. coli,* por tratamiento con luz ultravioleta (*E. coli,* UV-1 y UV-5), una mutante DR de *E. coli* por tratamiento con uretano (*E. coli,* U-12) y dos mutantes DR de *S. aureus* (*S. aureus,* UV-5 y UV-6).

En el *cuadro 1* se muestran los Q_{O_2} de las razas citadas, así como los de las razas originales, en ausencia y presencia de KCN, 0,01M.

(*) Actividad catalásica, según Euler y Josephson (3).

Cada una de las cifras que aparecen en el *cuadro 1* es la media aritmética de 3 determinaciones.

Cuadro 1. Actividad respiratoria de las razas de E. coli *y* S. aureus *usadas, en ausencia y presencia de KCN 0,01M*

Razas	Q_{O_2}	Con KCN 0,01M Q_{O_2}
E. coli, SS-47$_1$	236,0	29,5
E. coli, UV-1	104,7	60,0
E. coli UV-5	123,4	88,6
E. coli, U-12	92,0	37,4
S. aureus, C_4	142,8	13,0
S. aureus, UV-5	63,0	51,0
S. aureus, UV-6	58,4	31,9

Actividad catalásica de las razas ensayadas

En el *cuadro 2* se expone la actividad catalásica de cada una de las razas usadas, así como el tanto por ciento de disminución de aquélla en las mutantes DR. Cada uno de los resultados es la media de 5 determinaciones.

Cuadro 2. Actividad catalásica de las razas de E. coli *y* S. aureus *usadas y de sus mutantes DR*

Razas	*Kat. f.*	Disminución *Kat. f.* Porcentaje
E. coli, SS-47$_1$	14,44	
E. coli, UV-1	7,05	51,2
E. coli UV-5	7,70	46,7
E. coli, U-12	6,62	54,2
S. aureus, C_4	12,98	
S. aureus, UV-5	6,37	50,9
S. aureus, UV-6	5,71	56,0

DISCUSION

Es evidente, a la vista de los resultados expuestos en el *cuadro 2,* que las mutantes de *E. coli* y *S. aureus* estudiadas tienen una actividad catalásica menor que la de las razas originales. Como, de otra parte, el carácter primario, fundamental, que diferencia estas mutantes de las razas de que proceden, es la disminución irreversible de su actividad respiratoria, llegamos a la conclusión de que la deficiencia respiratoria en bacterias, al menos en las especies estudiadas, va acompañada, de manera constante, de una disminución de su actividad catalásica.

RESUMEN

Mediante tratamientos con luz ultravioleta y uretano, se han obtenido 3 mutantes de *Escherichia coli* y 2 de *Staphylococcus aureus* con deficiencia respiratoria. Ha sido determinado el contenido en catalasa de estas mutantes y de las razas originales de que proceden, encontrándose que la actividad catalásica de las razas con deficiencia respiratoria es, de manera constante, menor que la de las razas originales. La pérdida en actividad catalásica osciló, en el caso de las mutantes de *E. coli,* entre 46,7-54,2 por ciento, y en el caso de las mutantes de *S. aureus,* entre 50,9-56,0 por ciento.

SUMMARY

Three mutants of *Escherichia coli* with impaired respiration, and 2 of *Staphylococcus aureus,* have been obtained by irradiation with ultraviolet and treatment with urethane. The contents of catalase in the bacterial cells with impaired respiration in compararison with those of their parents were stimated. The mutants with impaired respiration showed a smaller catalase activity than the parents strains. The damage in catalase activity was, for *E. coli* mutants, of 46.7-54.2 per cent and of 50.9-56.0 per cent for *S. aureus* mutants.

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UNIVERSIDAD DE BUENOS AIRES
FACULTAD DE FARMACIA Y BIOQUIMICA
CATEDRA DE MICROBIOLOGIA, INMUNOLOGIA Y SEROLOGIA

A STUDY OF THE BACTERIUM ASSOCIATED WITH AN OUTBREAK OF OEDEMA AMONGST GOLDFISH *(CARASSIUS CARASSIUS* VAR. *AURATUS)*

BY
D. A. CONROY

INTRODUCTION

During the course of an outbreak of a fatal disease amongst cold-water goldfish *(Carassius carassius* var. *auratus)* in the late September of 1959, a complete bacteriological examination was initiated to study the condition, and to determine if possible the causes. The present communication describes the organism isolated in this work, and seeks to correlate the findings with available information of fish diseases of a similar type.

The fish were maintained in an outdoor pool situated within the Home Counties of England. It was noted by the owner that the fish were dying for some undetermined reason, and on the subsequent death of further specimens, the bodies were at once removed from the water and taken to the laboratory. Directly upon their receipt, they were examined as described below, and it is calculated that not more than one hour elapsed between the death and subsequent laboratory examination of the fish.

MATERIALS AND EXPERIMENTAL METHODS

A complete examination of the external surface of the fish was made, careful attention being given to the occurrence of any abrasions caused by physical or biological agency. In the latter case, a search was made for

any lesion of fungal, helminth, or arthropod origin. The general appearance and overall condition of the flesh, fins, eyes, mouth, gills, and scales was noted.

The fish were thereafter opened by means of instruments sterilised by boiling for 30 min in a steriliser. Immediately prior to dissection, the external surface of the animal was liberally swabbed with a 1 : 1,000 solution of mercuric chloride, and the instruments when not actually in use were also covered with a swab of the same fluid. Mercuric chloride was preferred to the 0.5 per cent brilliant green or crystal violet in 50 per cent alcohol recommended by Stewart (38) because of the subsequent need to effect a careful examination of the flesh. However, this workers method of sterilising the ventral surface of the fish by flaming with alcohol was adopted before making the initial cut, as this seemed preferable to searing with the blade of a spatula. A number of sterile Petri dishes were at hand, in order to cover the body of the fish when once opened, and to conserve any organs removed for the detailed bacteriological or histopathological examinations which might be thought necessary.

A pair of sharp-pointed dissecting scissors was used to effect the initial cut into the body cavity. The point of the scissors was inserted carefully into the region of the anal opening, or vent, and a series of cuts made anteriorly along the mid-ventral line. Subsequently the entire body wall of the animal was removed on one side to expose the internal organs completely. The lid of a large Petri dish was immediately placed over the fish to prevent any airborne contamination.

By means of a hand lens (magnification approximately $\times$ 8), a cursory examination of the organs was made in an effort to detect any obvious abnormalities. An alcohol-flamed mounted needle was used to move organs where necessary, and the whole proceedure was carried out under the Petri dish lid.

The operculum was completely removed, and the gills examined, with particular attention being given to fungal hyphae or trematodes. Smears, which were subsequently stained by Gram's stain, were then taken from the gills, heart blood, liver, kidney, and the fluid found to be present in the peritoneal cavity. Cultures were made into peptone water from the same organs, and incubated at 25 °C.

Following 24 h incubation, the peptone water cultures were examined

microscopically, and plated onto nutrient agar plates, which were further incubated at 25 °C.

The colonies developing on nutrient agar were again examined microscopically, and a typical colony removed and purified by passage through peptone water and nutrient agar. From the purified culture, the following media were inoculated:

a) Peptone water sugars, with phenol red indicator, containing 1 per cent lactose, dextrose, saccharose, maltose, mannitol, rhamnose, salicin, dulcitol, arabinose, inositol, galactose, xylose, and adonitol.
b) Christensen's urea agar.
c) Clark and Lubs' medium.
d) Nutrient gelatin.
e) Blood agar containing 5 per cent oxalated bovine blood.
f) Lead acetate agar.
g) Litmus milk.
h) Lab-lemco broth "Oxoid".
i) Nitrate broth.

These media were incubated at 25 °C for a total of 7 days, during which period daily observations were made to note any reactions which may have occurred.

From an active peptone water culture, duplicate series of nutrient agar slopes and nutrient broths were inoculated, and the media were placed at temperatures of 4 °C (laboratory refrigerator), 17-19 °C (room temperature of the laboratory), 25 °C, and 35 °C, to determine the optimum temperature of the organism.

Further tests were initiated to study other of the properties of the organism, and the following media were used for this purpose:

a) McClung and Toabes' egg yolk agar (23).
b) Nutrient broth distributed into an Einhorn saccharometer.
c) Nutrient agar containing 2 per cent starch.

The tests employed for the detection of indole, acetylmethylcarbinol, hydrolysis of starch, production of catalase etc., follow standard bacteriological procedures, and will be briefly referred to in each case under "Results".

Examination of the organism for sporulation, encapsulation, motility and flagellation were likewise made according to well known procedures.

In order to determine the pathogenicity of the isolate, both to goldfish and to white mice, the methods described below were adopted.

a) Goldfish

Small 7-10 cm long goldfish were purchased from a reputable dealer, and were selected personally on a basis of their general appearance. Every effort was made to choose healthy fish, and special attention was paid to the condition of the eyes and scales, and to the rigidity of the fins, these characters being most commonly noted by fishbreeders in the purchase of healthy stock. Extreme caution was used in the transference of the fish from tank to tank, a fine meshed net being used for this purpose.

In the laboratory, the fish were placed into aquaria which had been previously disinfected with a strong solution of permanganate of potash. A total of 24 fish were purchased in all, and these were divided up into 4 lots of 6 each, and housed in separate tanks labelled "A", "B", "C", and "negative control". All these tanks had been provided with efficient artificial aeration, and strands of green plants such as *Elodea sp.* and *Myriophyllum sp.* added. Aquatic molluscs of the genera *Limnea* and *Planorbis* were added in the proportion of one of each genus per tank. Every effort was made to provide a balanced community. Food was added once daily, equal proportions of the two proprietary brands "Meta" and "Vega" being used for this purpose, and when possible, live food in the form of *Dephnia sp.* was also supplied. The fish were allowed to acclimatise themselves to their new environment for a period of 7 days before the commencement of the experiment and in this time careful note was taken of the presence of any lassitude, or similar conditions.

The fish from tank "A" each received 0,2 ml of a 24 h peptone water culture by the intraperitoneal route. A tuberculin syringe was found to be useful in this way, and the simple technique used to effect this without damage to the fish has been described in detail by Conroy and Hughes (8).

The fish from tank "B" were given aproximately 0,2 ml of a similar culture to that used above. This was administered *per os*, the procedure used being to immobilise the fish by holding gently yet firmly in a net. The mouth is held uppermost, and the culture contained in a sterile

Pasteur pipette is carefully passed into the buccal cavity. To accomplish this, the fine point of the pipette is inserted cautiously into the mouth, and culture slowly released. An interval of approximately 5 seconds should preferably be allowed to elapse before returning the fish to the water, as this permits optimal diffusion of the culture into the anterior part of the alimentary tract.

To the water of tank "C" was added a peptone water culture of the organism to a concentration of approximately 25 ml/l of water. This was to observe the effect of the organism upon the fish when the bacterium itself was known to be present in the water.

The tank labelled "negative control" was allowed to remain free from the isolate, and the fish contained therein were used as a source of "reference material" for comparison purposes.

All three tanks remained at room temperature for a total of 30 days, under normal conditions of lighting and heating. During this period daily observations were made upon the fish, and on the appearance of any conditions resembling those exhibited by the fish originally studied, the animal affected was removed and treated as described above. Cultures were likewise taken from the bodily organs.

b) White mice

Ten white mice of the "Swiss" strain, each weighing 18-22 g, were used for this study. Six of them were inoculated with 0,2 ml of a peptone water culture; the remainder with 0,5 ml of the same inoculum. All were injected by the intraperitoneal route, and differentiated by means of distinctive colours. They were maintained in mouse cages of a standard pattern, and fed with oats, bran, and lettuce.

Daily blood examinations were made, the sample being removed from the caudal vein by means of a fine bore hypodermic needle, and transferred to a wax-lined tube to prevent clotting.

Smears were prepared and stained by Leishmann's stain, examination being made with the oil immersión objetive. Further smears were stained with Jenner-Giemsa, in order to study the morphology of the cells.

RESULTS

Post mortem examination of the fish

The fish received in the first instance were examined as described under "Materials and Experimental Methods". No apparent injuries were noted in any of the fish examined, the fins were in good condition, showed no signs of having recently been subjected to fin rot or any allied conditions, and discoloration was not observed. The eyes were quite bright, and not extended; the scales appeared normal in all respects; and the skin was firm, of normal coloration and smell. No external parasites or their lesions were detected.

The mouths and opercula of the fish when received into the laboratory were open in all cases, giving the appearance of suffocation. This at once suggested that a chemical agency my have brought about death through its being toxic to the fish, and a sample of the pond water was accordingly obtained and submitted to chemical examination. As the vicinity of the pool had been sprayed with weed-killing compounds during the course of the Summer, it was feared that the dust from these may have entered the water, and provoked a toxic condition in the fish. As was subsequently demonstrated however, the chemical findings for toxic residues were negative. In a similar fashion, ectoparasitic trematodes e. g. *Dactylogyrus sp,* were not detected in a careful examination of the gill *Lamellae.*

The body of each fish, on the other hand, presented a markedly abnormal picture. The organs and systems will be dealt with one by one for the sake of clarity. Each body was very distended, the swelling being quite firm and not yielding to the touch.

a) Digestive system

The stomach showed no external abnormalities itself, but from the duodenum to the anus, the entire alimentary tract was heavily inflated with gas pockets, and swollen with liquid. The overall colour was that of olive oil, interspersed with occasional bloody tinges.

b) Kidney

This organ was surrounded by a malodorous fluid with a very oily consistency, and of a clear, slightly yellow appearance.

c) Heart

In the heart, the blood appeared slightly haemolysed. To avoid any erroneous conclusions being drawn from this, a smear was immediately prepared and stained by Gram's stain. Examination showed the presence of Gram negative rods in fair numbers.

d) Swim bladder

This appeared to be abnormally distended.

e) Fluid within peritoneum

On opening the peritoneal cavity, a large quantity of the same fluid as described under "kidney" was encountered. The amount of fluid was such that on making the first incision into the body, the fluid was expelled rapidly, and spread over the surface of the laboratory work bench for some considerable distance. No bacteria were actually detected in the microscopical preparation made, but it is felt that this may be due in part to the technical difficulties encountered in the preparation of the smear, as subsequent cultures from the same fluid were all positive. A foul odour emanated from the fluid.

f) Gills

The gills were the most noticeable of the organs affected. The *lamellae* were of a grey-brown colour, and were excessively laden with mucus. Smears made from them showed large numbers of the Gram negative rod to be present.

g) Body wall of the peritoneum

The internal surface of the body wall had a normal appearance, following careful removal of the overlying fluid with a sterile cotton wool swab. The tissue was firm, and when a portion of the muscle was

removed aseptically by excision with Bog needles, was found to be negative on culture.

The organism found occurring in the fish examined was present in pure culture. The isolate was further purified however by successive plating and sub-culturing prior to the performance of any diagnostic tests, and the results contained herein were obtained from the culture taken from the gills. Replicate properties were seen to exist in cultures from the heart blood and the fluid from the peritoneum, andt there is little doubt that the same organism was present throughout.

Characteristics of the isolate

The organism having been purified by succesive plating and subculture, was found to possess the following characteristics.

Morphology

Gram negative rods, which were seen to occur singly, in pairs, or occasionally in chains of up to four. The appearance of the organism is shown in *Figure 1*. The average size, when measured with a micrometer, was $0.7 \times 1.4\ \mu$. No spores were seen when 7 day cultures were subjected to the Schaeffer and Fulton modification of the Wirtz stain. The use of Hiss's stain showed that the organism was not encapsulated. Motility was evident from hanging drop preparations made from 18 h peptone water cultures, and one polar flagellum was seen to be present when stained by Kirkpatrick's stain.

Gelatin stab

Gelatin was readily liquefied, the study being repeated a number of times in confirmation. The mode of liquefaction appeared to be initially infundibuliform, and a powdery white sediment came to be formed on prolonged incubation. No discoloration of the medium was observed.

Agar colonies

The colonies produced on nutrient agar were circular, and possessed entire edges. The form was raised to slightly convex, and the colonies were translucent on original culture, becoming slightly opaque later. The form of growth is shown in *Figure 2.*

Agar slant

Growth was heavy, smooth, and glistening. There was no discoloration of the medium.

Nutrient agar stab

The organism grew well along the line of the stab, although growth was not good towards the basal portion of the medium. It is therefore assumed that the organism is obligately an aerobe, but that it possesses the ability to grow under microaerophilic conditions.

Peptone water

Heavy and turbid growth within 24 h. After 3 days a fine flocculent sediment is formed, and a membranous pellicle may be visible.

Lab-lemco broth

Growth was as for peptone water. A light fluorescence was seen after 4-5 days incubation at 25 °C. This phenomenon appears to be somewhat inconstant.

Litmus milk

No marked change.

Metabolism

Indole produced from peptone water, when tested by both Kovac's and Ehrlich's methods. It is surprising that indole should be produced, for this is not characteristic of *Pseudomonas sp.*

Catalase is produced from growth in nutrient broth, large volumes of oxygen being evolved on the addition of 1 ml of 10 volume hydrogen peroxide.

Hydrogen sulphide is produced in small amounts when the organism is grown in lead acetate agar.

Citrate may be used as the sole source of carbon, as evidenced by growth in Koser's citrate medium.

Urease is not produced when grown on Christensen's medium.

Ammonia is produced from peptone water, giving a strongly positive reaction with freshly prepared Nessler's reagent.

Lecithinase is produced when the organism is grown on McClung and Toabes' medium.

Methyl red negative.

Acetylmethylcarbinol is seen to be formed (in what appears to be small quantities) in a broth containing glucose such as Clark and Lubs' medium. The Voges-Proskauer test, and the Barritt modification of the same were both employed to study this reaction.

Starch is hydrolysed to a small degree, giving a typical reaction with Lugol's iodine, when the latter was used to flood the plate. The reaction is shown in *Figure 3*.

Acid, but no gas, is produced from dextrose, saccharose, maltose, and mannitol. Slow acid production from galactose. Lactose, rhamnose, arabinose, dulcitol, xylose, salicin, adonitol, and inositol are not attacked. It is interesting to note that where the peptone water sugars are left at room temperature for a few days, an alkaline reaction comes to be formed (evidenced by a red colour) on the surface of the medium. It is assumed that this reaction arises from the production of ammonia by the organism when growing in heavy concentration in the form of a pellicle. Of further interest is the fact that maltose should be attacked, in view of the statement by Dowson (12), that this sugar is rarely attacked by pseudomonads.

Haemolysis of the beta-type is produced on agar containing 5 per cent oxalated bovine blood. These zones of haemolysis are extremely clear, and are shown to advantage in *Figures 4-5*. The reaction is produced within 30 h incubation at 25 °C.

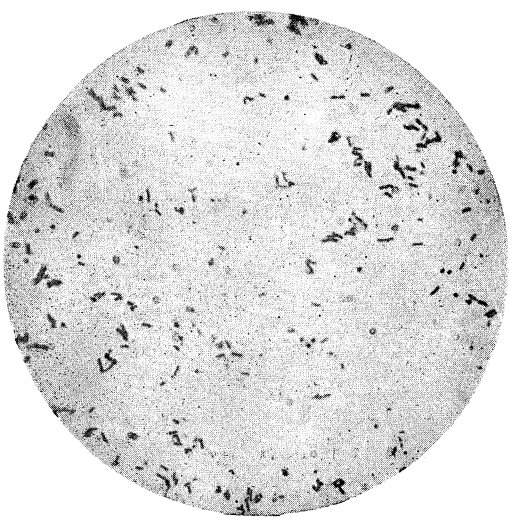

Figure 1. Microscopical appearance of the organism. ×750

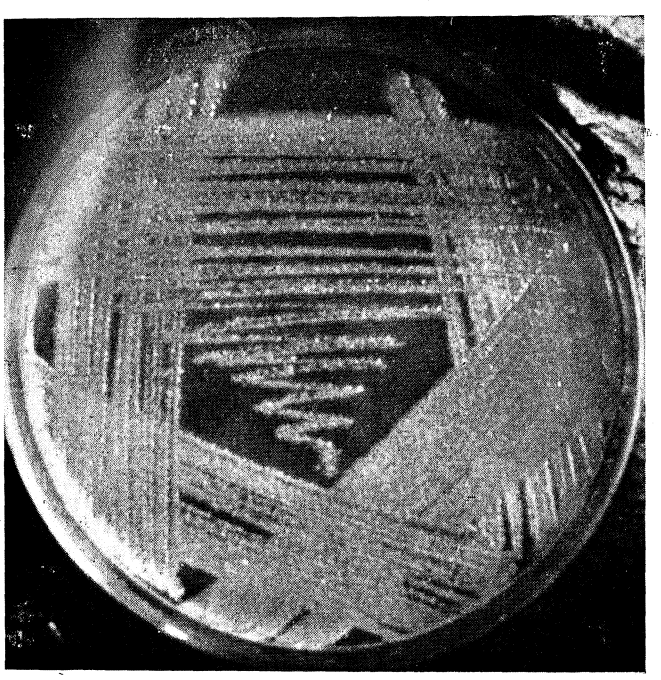

Figure 2. Growth upon nutrient agar

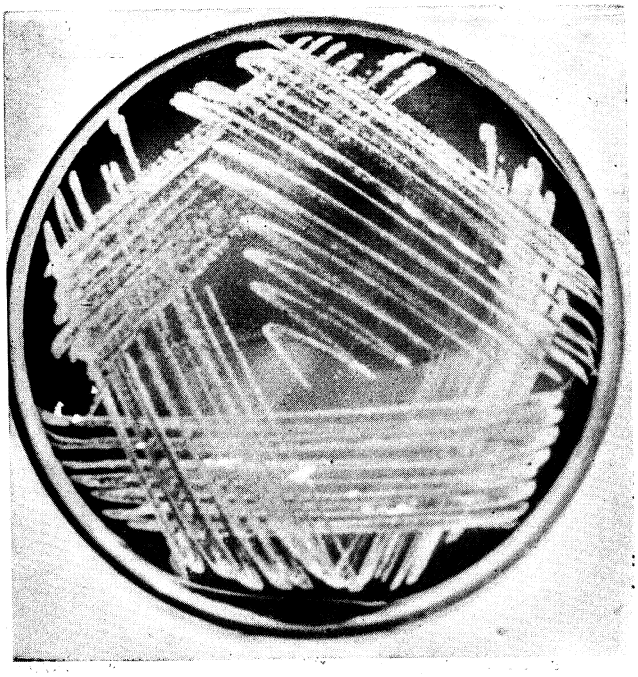

Figure 3. Growth upon starch agar, and reaction with Lugol's iodine

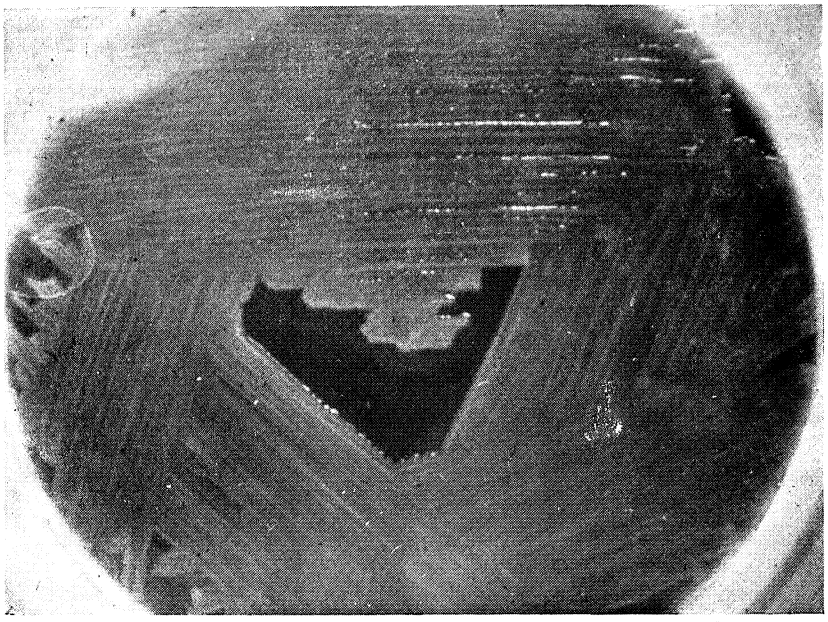

Figure 4. Production of beta-haemolysis on blood agar

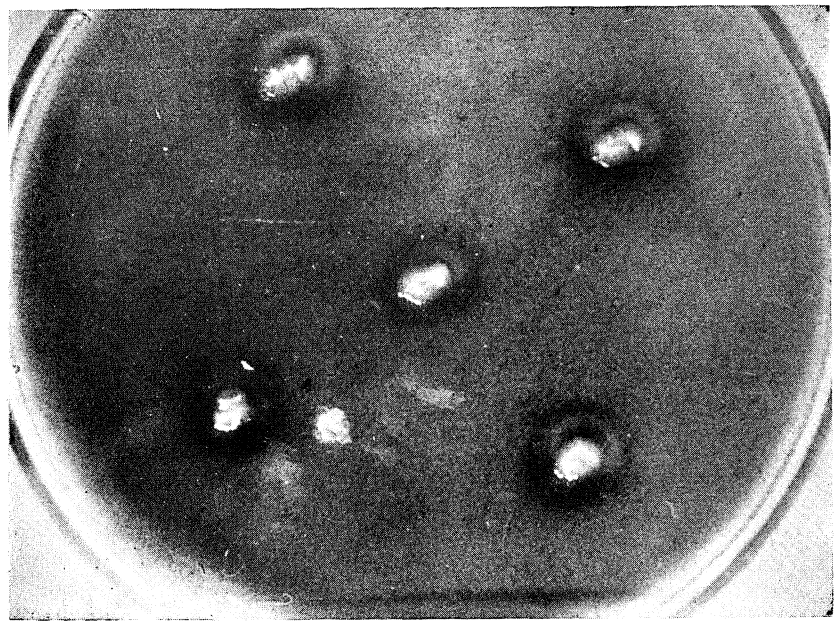

Figure 5. Beta-haemolysis developing from stab

Pathogenicity

The pathogenicity of the organism to goldfish was given a somewhat more detailed study than was its action upon white mice. Of each of the groups of 6 fish in the 4 tanks, the following results were recorded:

Tank "A"

Oedematous conditions of a slight degree were developed to a variable extent in three of the fish inoculated via the intraperitoneal route. The most advanced case was autopsied as described above, and the bacterium was isolated from the small amount of fluid formed within the peritoneal cavity, the heart blood, and the gill surface. Cultures from the kidney, liver, and body wall were negative. To effect the autopsy, the fish was placed in a beaker of water, and anaesthetised by adding urethane to produce a 3 : 100,000 concentration, as recommended by Pentelow (26). The conditions which had developed resembled those seen in the original specimens to a modified degree. Marked swelling of the belly was present, typical of a slight fish oedema, and the body cavity contained fluid similar to that found in the originals, save that the odour was not present. This condition had developed within 14 days after inoculation, and had become more pronounced on the 17th day. The two other fish whose symptoms were not so pronounced were allowed to remain in the tank, but did not seem to worsen, and the condition had not led to fatality in 30 days. A photograph was taken of the fish used for post-mortem, both before and after autopsy, but it is regretted that neither photo came out satisfactorily, and unfortunately cannot be reproduced here. All the three other fish remained unaffected and apparently healthy.

Tank "B"

None of the fish in tank "B" developed what are termed typical symptoms of oedema. One specimen was found in a state of lassitude, but this symptom rapidly disappeared following the addition of sulphate of magnesia.

Tank "C"

The fish in this tank remained completely immune from any symptoms of ill health. It was concluded that the presence of the organism in heavy quantity in the water had not had any deleterious effect.

The negative controls remained perfectly healthy, as one might expect. In none of the fish were symptoms of photophobia noted, and loss of appetite was of temporary occurrence in the constipated individual only. The presence of the symptoms was seen by Besse (2) in the bacterial dropsy of trout (*Salmo iridaeus*) which he studied.

As regards the pathogenicity of the organism to white mice, it would appear from the results obtained that the organism possesses no pathogenic powers towards these animals. The blood examinations, however, showed an increase in the number of monocytes between the 2nd and the 5th day following inoculation. The percentage of monocytes, calculated on a basis of the total and differential leucocyte counts, rose to 9 per cent on the 3rd day, and had become reduced to the normal 2-3 per cent of these animals by the 5th day. Careful examination of the smears by the Jenner-Giemsa technique of staining failed to show conclusively the presence of typical bacteria within the monocyte cytoplasm.

Temperature relationships

The organism is capable of growth at all of the temperatures given above. However, the optimum temperature for growth appears to be 25 °C, when based on the degree of turbidity produced in peptone water.

Discussion

Bacterial diseases of fish are of common and wide occurrence in almost all parts of the world. Relatively little work yet been done on the subject however, although of the genera and species so far implicated as causal of these diseases, many belong to the family *Pseudomonadaceae*. An exhaustive and comprehensive study of the subject has been made by van Duijn (40), and a great deal of valuable information was obtained from the monograph of this author.

The symptoms shown by the infected fish as described in this communication, resemble those given in the literature for the classical oedema of cyprinids. Preliminary studies, based upon the pathological findings on dissection, and the isolation of motile gram negative rods from the exudate and organs, led to the organism being provisionally classified as *Pseudomonas punctata* Zimmermann. Subsequent work has caused this classification to be revised. Infections of members of the family *Cyprinidae*, to which the goldfish apertains, by *P. punctata* have the following characteristics, according to van Duijn (40):

The belly of the fish is swollen with liquid of a watery appearance. In addition, the bodily organs become infected in some instances. The findings of other European workers are considered, and these are of the opinion that two distinct forms of the disease exist. These distinct types are termed the Polish and the German forms respectively, and they seem to vary in regard to their pathogenic properties. The Polish form produces ulcerous lesions which are not found in the other, whereas the German form produces large amounts of intraperitoneal fluid, causing the belly to become greatly distended. When the disease occurs amongst aquarium fish, scale protusion may be present in the afflicted individuals.

On dissection, the fish emit a strong and characteristic odour, and inflammation of the bodily organs may be observed. It will have been noted from the results given earlier in this work, that the symptoms indeed resemble very closely those given by van Duijn for infections caused by *P. punctata*. Also, the symptoms resemble those given by Snieszko (33) for fish diseases caused by members of the genus *Pseudomonas* and the related genus *Aeromonas*. The condition is considered by Snieszko to be septicaemic, allowing the organism responsible to be readily isolated from the blood and internal organs. Furthermore, it is stated that exopthalmus may occur, and that internally a purulent fluid is present. Whilst exopthalmus was not present in thist case, the purulent fluid was very much in evidence. The lower intestine and vent are also inflamed and swollen, a further symptom present in this case. Mortality rates are said to vary, according to the degree of infection, and the resistence of the individual fish. From the symptomatic basis therefore, the writer concludes that the condition described in this work is of a type similar to that described by van Duijn and Snieszko for piscine dropsy.

Jacobs (17) also cites *Pseudomonas sp.* as being causative agents of fish dropsy.

From blueback salmon (*Oncorhynchus nerka*), Rucker (29) isolated a pseudomonad and a streptomycete evisting together. The fish which he examined showed signs of haemorrhagia coupled with disintegration of the kidneys and spleens. This organism was also cultivated from the bodily organs, and was provisionally classified as *Pseudomonas punctata*, although Rucker considered at the time the possibility of its being a new species. He was able to prove by the inoculation of pure cultures into healthy fish, that it caused peritonitis, and that death frequently ensued from septicaemia on about the 12th day after inoculation. This work lent further support to the provisional classification of this present isolate as *P. punctata.*

Further more detailed studies have shown that this classification cannot be applied to the organism isolated in this case, even though many of the properties of the latter are in broad agreement with the characteristics laid down in "Bergey's Manual of Determinative Bacteriology" (1) for *Pseudomonas punctata.* The cells are Gram negative, motile by means of a single polar flagellum, occur singly, in pairs, or in chains. Their average size is $0.7 \times 1.4\mu$. Gelatin is liquefied, hydrogen sulphide is produced, indole is produced, and ammonia formed from peptone. The optimum temperature appears to be 25 °C, all of which details are given for *P. punctata.* Schaperclaus, quoted by van Duijn (40), considers that several distinct types of this organism may be found occurring naturally, and they differ slightly in their pathogenic properties. He found the typical *Pseudomonas punctata* variety to be distributed widely in waters, and furthermore that it was harmless. On the other hand, the variety causing cyprinid oedema, he termed *Pseudomonas punctata forma ascitae.* However, van Duijn questions the validity of this differentiation, as he does not consider that the types described by Schaperclaus are constant, and disagrees with their being termed distinct varieties.

The principal feature of the isolate which would tend to distinguish it from the true *P. punctata* is the light production of fluorescence in evidence in lab-lemco broth, and the fact that the other ferments sugars with vigorous production of acid and gas. According to van Duijn (41) these factors determine definitely that its identity is not *P. punctata.*

He suggested that the organism might be a strain of *Pseudomonas granulata,* making reference to the work of Brunner and Striegel (4). The basis of this identification is the variable ability of that organism to produce fluorescence, the absence of fluorescence not denying specific identity. When fluorescence is absent, the species is recorded as having the power to ferment sugars with the production of acid and gas, glucose alone being fermented in the fluorescing strain. The apparent inability of *P. granulata* to attack mammalian blood, although capable of producing haemolysis on fish blood, is contrasted to the well-defined beta-haemolysis produced on bovine blood by the species isolated in this work. It was pointed out by van Duijn (41) that in the study of fish pathogens, one should not confine oneself solely to a study of the organisms reactions on media intended for mammalian pathogens, but that media containing fish blood should also be used. Unfortunately, it was not possible to effect a study of the action of this isolate upon goldfish blood agar, bu it is pertinent to cite the work of Caldwell and Ryerson (5), who studied the characteristics of *Pseudomonas reptilivorous* (*P. reptilivora*) which they isolated from horned lizards (*Phrynosoma solare*) and Gila monsters (*Heloderma suspectum*). Working with 2.5 per cent rabbit blood and 1.5 per cent Gila monster blood agar plater, they were able to show that the rabbit blood was markedly haemolysed within 18 h, and that the Gila monster blood was only very slightly haemolysed after 24 h incubation. Likewise, their isolate was found to be present in both the heart blood and peritoneal fluid. Therefore it is felt that there is sufficient precedence to allow the study of the organisms action upon fish blood agar to be foregone, as Liu (19) has also shown that haemolysis is of more common ocurrence amongst the pseudomonads than was previously believed. Additionally, he concluded that *P. reptilivora* possesssed poor powers of haemolysin production. Therefore from the work of these authors, it appears possible to correlate the haemolysis shown by the isolate herein described with its inclusion in the genus *Pseudomonas.* It is of interest to note that Snieszko (33) considers that little or no leucocytic response occurs with *Aeromonas salmonicida* infections in fish. Furthermore, it was suggested by van Duijn (41) that the organism may be a strain of *P. putida.* Carp (*Cyprinus sp.*) and tench (*Tinca sp.*) are often infected by *P. putida,* and the disease is termed *Purpura cyprinorum* or "red pest" by van Duijn (40).

According to that worker, the symptoms produced amongst the infected individuals are a dark coloration of the ventral skin, haemorrhages, and necrosis of the gills, and red coloration of the ventral, anal, and caudal fins. Internally, there may be found inflammation of the intestine, and occasionally bloody tinges are evident. It is emphasised that the disease is highly contagious, and that the entire fish population may be rapidly infected if adequate precautionary measures are not adopted. Two strains of. *P. putida* may be responsible, a nonmotile *P. putida forma Davidii,* and a motile (1-6 flagella) *P. putida forma cyprinicida.*

However, comparison of the present isolate with a known strain of *P. putida* showed that the former differed from the latter both in its liquefaction of gelatin, and in its production of indole. Moreover, the type culture of *P. putida* studied gave a distinct odour of trimethy lamine, which differed markedly from the odour of the goldfish organism, though it must be pointed out that this also was strongly unpleasant.

Amongst the other pseudomonads associated with red pest disease, van Duijn lists *Pseudomonas Plehniae,* which he says is closely related to *P. punctata.* Furthermore, *P. punctata* has been found in cases of red pest disease of eels (*Angilla sp.*), where it gives rise to a cutaneous ulceration. The strain responsible for this condition has been termed. *P. punctata forma sarcowiensis* by Schaperclaus, but here again van Duijn questions the validity of this nomenclature in view of the fact that he considers the varieties of *P. punctata* to be inconstant.

What is immediately apparent from the condition described in this thesis and those present in infections caused by *P. punctata, P. putida,* and *Aeromonas sp.*, is that the symptoms are parallel. There would therefore appear to be present little doubt that a typical condition of this nature is being described, although the organism isolated may differ slightly from others asosciated with this type of disease.

Of the true fluorescent pseudomonads displaying a pathogenicity to fish, *Pseudomonas fluorescens* itself has received the atention of many investigators. An excellent description of a disease of labyrinth fish (*Anabantidae*) caused by this organism, is given by van Duijn (40). Schaperclaus found that this species had given rise to a secondary ulceration in eels (*Anguilla sp.*) which had been injured, but van Duijn failed to find any evidence of necrosis or ulceration in his investigations with labyrinths. A similar disease is known to occur in

dace (*Leuciscus leuciscus*) where the infecting agent may be *P. fluorescens, P. putida,* or *Proteus vulgaris. P. fluorescens* has ben shown to be present in large numbers in the slime of the haddock (*Gadus aeglefinus*) and of the halibut (*Hippoglossus vulgaris*).

From marine fish, Hodgkiss and Shewan (16) found an infection of plaice (*Pleuranectes platessa*) to be caused by a species of pseudomonad. This work is especially of interest in that many of the characteristics described by these two workers for their isolate, resemble closely the salient features of the organism whose properties are currently being considered. The plaice examined by them had a lesion on the skin, which when opened, gave forth a stream or pus-like fluid under pressure. The bacterium was seen to be present in this fluid. Fluorescence was detected on agar plates, and acid was produced in dextrose, saccharose, mannitol, and maltose. Lactose and dulcitol were not attacked. Starch was hydrolysed, and gelatin liquefied. Furthermore, both indole and ammonia were produced from peptone water. And alpha-haemolysis was produced on horse blood agar, and the organism was shown to be non-pathogenic for white mice. However, the methyl red test was positive, and the Voges-Proskauer reaction negative, which is somewhat in contrast to these reactions of the isolate described herein. The organism isolated by Hodgkiss and Shewan is of marine origin, and although sea water was found not to be an essential factor for its growth, was considered by them to resemble *Pseudomonas ichthyodermis.* This latter organism is stated by ZoBell (42) to cause an infectious dermatitis of marine fishes.

The work of Rhodes (27) on *P. fluorescens* has provided an ample description of the more important features of that species, and was used to provide a concise summary of the typical characteristics of the species in in attempt to identify the present isolate as a strain of *P. fluorescens.* The production of indole, hydrogen sulphide, acetylmethylcarbinol and haemolysis are thought to distinghish the organism isolated from the true *P. fluorescens* however, as also does the apparent inability to produce fluorescence readily upon ordinary laboratory media. It is of interest to recall that many of Rhodes isolates were themselves obtained from ornamental ponds and similar situations, though no known fish pathogens of the genus *Pseudomonas* were studied (28). Indeed, of the species considered to be pathogenic to poikilothermic animals, *P. hydrophila* she immediately classified as a member of the genus *Aeromonas.*

The incorporation of several pseudomonads into this new genus *Aeromonas* (Kluyver and Van Niel) has presented a further complication to the accurate identification of the species isolated. According to "Bergey's Manual" (1), the salient features of this genus are:

"Short (rarely more than 3 μ), rod-shaped cells. Motile by means of polar flagella, usually monotrichous; occasionally nonmotile. Gram negative. Heterotrophic, oxidising various organic compounds. Carbohydrates fermented with the production of carbon dioxide, hydrogen, and 2,3-butylene glycol. Methyl red negative, slow or no fermentation of lactose. The majority of the species thus far described are from water or are known to be pathogenic to marine or freshwater animals such as fish and amphibians."

"Physiologically these organisms appear to be identical with certain species found within the family *Enterobacteriaceae*. The chief difference between the species found in *Aeromonas* and those in *Paracolobactrum* (Borman, Stuart, and Wheeler) are found in the arrangement of their flagella, in the less active fermentation of carbohydrates by the former, and in their pathogenicity."

"The type species is *Aeromonas liquefaciens* (Beijerinck) Kluyver and van Niel."

Pseudomonas punctata itself has now been included in this genus, so that it is now known officially as *Aeromonas punctata*. For reasons of clarity, and to avoid any unnecessary misunderstanding however, the older generic name has been retained throughout this thesis, even if this may be taxonomically incorrect. The merits of the proposed inclusion of *Proteus* (*Pseudomonas*) *hydrophilus* in the genus *Aeromonas*, have been amply considered by Stanier (35). As that worker also points out, should the suggested relationship between *Proteus hydrophilus* (*Aeromonas hydrophila*) and *Pseudomonas punctata* be ultimately proven, the latter specific name would have priority. The new genus *Aeromonas* has received a detailed consideration by many authors, and a wide variety of genera and species are now embraced within the one species *Aeromonas liquefaciens* by Snieszko (33). It is not possible to do more than consider briefly the possibility of including the organism under discussion within this genus, for the author is not a bacterial taxonomist, and the difficulties in making such a "label" are enormously complex (34).

Snieszko (33) lists infections of abdominal dropsy, haemorrhagic septicaemia, red sore disease, and red mouth disease as being caused by "pseùdomonas-like bacteria which are now classified as belonging to the genus *Aeromonas.*" He further says that diseases arising from "the related genus *Pseudomonas* are less common", though as has been shown earlier in this work, there are several well authenticated cases in the literature. The four diseases listed above are considered to arise as a result of infection by *A. liquefaciens,* or by bacteria closely related to it, though it is noted with interest that similar diseases and conditions may be brought about by pseudomonads producing fluorescence on special media. The diseases are collectively designated as being "septicaemic", which allows the organisms responsible to be readily isolated from the blood. This pattern is followed in the infection described in this thesis. As a presumptive means of identification, Snieszko suggests that the following features be of diagnostic significance:

a) Gram negative.

b) Motile.

c) Single polar flagellum.

d) Growth from 10-35 °C.

e) Gelatin liquefied.

f) Acid and gas from mannitol, dextrose, and saccharose; no action (or slow action) with lactose.

The similarity between the above and the goldfish isolate is marked, with the solitary exception that the former produce gas in carbohydrate media.

The characteristics which therefore tend to distinguish the isolate from the *A. liquefaciens* group are its ability under some undetermined condition to produce fluorescence in lab-lemco broth, its lack of pathogenicity to laboratory mice, and its reactions with the sugars. It must be noted in the last respect, that the observations of Stein *et alia* (37) were that many species of *Pseudomonas* do in fact possess the ability to produce carbon dioxide from simple sugars when grown in media with Eldridge tubes.

The haemolytic properties of the organism must also be given due consideration, as this characteristic is not of particularly wide distribution amongst members of the genus *Pseudomonas.*

Guthrie and Hitchner (15), working with organisms isolated from "red leg disease" in frogs, found that there were but few significant differences between their isolate and *P. punctata*. These workers noted the monotrichous flagellation, the inability to produce ammonia from urea, and the production of acid and gas from mannitol by their own organism. Kulp and Borden (18) also were able to corroborate the work of earlier authors by showing that *Proteus hydrophilus* (*Aeromonas hydrophila*) produces gas from carbohydrates. Therefore, although the symptoms displayed by the goldfish isolate are in basic accordance with those described by Snieszko (33) and van Duijn (40) for *P. punctata* (*Aeromonas punctata* or *A. liquefaciens*), the apparent ability to produce a slight fluorescence in lab-lemco broth is taken as evidence of its adherence to the group of pseudomonads known as the "fluorescent pseudomonads", even though fluorescence when produced is not strong.

The difficulties of identification within the fluorescent pseudomonads is again difficult, as has been noted by Clara (6), Seleen and Stark (30), Gaby (14), and Rhodes (27). Gaby (14) considered that the production of acid from saccharose should automatically exclude an organism from the genus *Pseudomonas*, but it is felt that the widely accepted fluorescin production by pseudomonads (although the writer recalls having encountered a reference to a slight fluorescence being observed in cultures of *B. subtilis*), is of prior importance in identification. The work of Tobie (39) also supports this contention, for it is stated in his paper that the production of fluorescence is considered ground enough for inclusion amongst the pseudomonads. Certainly reference to Skerman (31) and Margni (20) would support this classification.

The organism will therefore be arbitrarily referred to as of the "*Pseudomonas fluorescens* species-group" proposed by Stanier (36), although fluorescence is not a distinctive feature, and is not consistently produced.

The suggested relationship between *P. fluorescens* and *P. putida* proposed by Clara (6), has more recently been discounted by Muñoz, *et alia* (24), on a serological basis, and therefore it is not possible to say whether the organism is related directly to, or is a strain of, any other known pseudomonad.

A description of the organism is given below, which follows in general terms the format of "Bergey's Manual (1).

Description of the pseudomonad isolated from a haemorrhagic and oedematous condition of goldfish

Gram negative rods, occurring singly, in pairs, or occasionally in chains. Average size 0.7 × 1.4 μ. Motile by means of a single polar flagellum. No endospore or capsule.

Gelatin stab: Infundibuliform liquefaction in a short time. No discoloration of the medium.

Agar colonies: Circular, with entire edges. Beading may be present. Form raised to convex, translucent, becoming slightly opaque.

Agar slope: Growth heavy, smooth and glistening. No discoloration of the medium.

Peptone water: Heavy and turbid growth within 24 h. A flocculent sediment is formed after 3 days, and a membranous pellicle may be visible.

Lab-lemco broth: Growth as for peptone water. A slight fluorescence may be produced within 4-5 days.

Litmus milk: No change.

Indole produced.

Ammonia produced from peptone.

Urease not produced.

Catalase produced.

Hydrogen sulphide produced.

Lecithinase produced.

Methylred negative.

Voges-Proskauer positive.

Citrate may be used as sole source of carbon.

Acid from dextrose, saccharose, maltose, and mannitol. Lactose, dulcitol, salicin, rhamnose, inositol, adonitol, xylose, and arabinose not attacked.

Slow acid production from galactose.

Starch hydrolysed.

Beta-haemolysis produced on 5 per cent ox blood agar.

A slight pathogenic effect may be demonstrated to goldfish. The organism is not pathogenic to white mice.

Aerobic, capable of growth under microaerophilic conditions.

Optimum temperature: 20 - 25 °C.

Source: Isolated from goldfish showing signs of oedema.

Habitat: Freshwater, presumably where animal life is found.

Having given thus a description of the organism isolated, it is next necessary to consider the way in which the infection was brought about in the first instante. Fish are considered to vary greatly in their resistance to infections by *P. punctata* and similar organisms. The work of Schaperclaus (quoted by van Duijn) showed that *P. punctata* is normally non-infective, and that fish in normal health remain free from infection, even in cases where diseased fish are present in the surrounding water. Indeed, a fish which was infected itself was found not to be infective to other fish. These findings are borne out largely by the results obtained in this present work, for the fish generally appeared to be unaffected by the organism except by direct intraperitoneal injection. Snieszko adds that overcrowding or rough handling may bring about outbreaks of haemorrhagic septicaemia and allied conditions, and such factors were deliberately avoided by the use of nets etc. when the pathogenicity tests were being performed in this case.

Several workers have shown that the disease occurs primarily in the Spring, following initial infection during the preceding Winter. Most certainly oedema is found to occur in the Spring and Summer, when the temperature of the water is more conductive to the rapid growth of the bacterium. It is thus of interest to recall that the condition described herein was found to occur in the Autumn season of the year, even though the infective organism may not be *P. punctata* as was originally thought. A hypothesis must therefore be formulated in an effort to explain this occurrence.

During the Winter season, fish are usually not feeding normally, and the infection may be contracted from the surrounding water. The

course of the disease is determined principally by the degree of resistance or susceptibility of the individual fish.

During the examinations carried out in this work on the diseased fish, no evidence was seen which would suggest attack by trematodes (monogenetic or digenetic), copepods, or leeches. These type of ectoparasites have been shown to contain the bacteria in their intestinal tracts (33). Of particular importance is the fact that *Gyrodactylus sp.* is specifically held to be responsible, as this is a well-known parasite of members of the *Cyprinidae.* Slack (32), working with *Bacillus salmonicida* (now *Aeromonas salmonicida*), from the brown trout (*Salmo trutta* found that the disease occurred each Spring and Summer. He considered from his experiments that there existed little evidence to incriminate *Gammarus pulex* as the possible vector, as had previously been thought by other workers. On the other hand, he was of the opinion that river water and silt suspensions should be held responsible. Although Jacobs (17) holds that such bacterial infections may be introduced by snails, infected water or contaminated nets, the presence of snails in the aquarium water did not appear to excercise any harmful effect during the course of this work, and therefore it is considered that these agents played no role as intermediate vectors.

Margolis (22), in a study of the bacterial contents of the intestine of bullheads (*Ameiuris nebulosus*) and trout (*Salvelinus fontinalis*) after starvation, found that the majority of fish possessed no intestinal bacteria. A pseudomonad was isolated in pure culture from the tract of the remaining fish. These results are similar to those obtained by Fischer (13) working with cod (*Gadus morrhua*). It is assumed from the results obtained in the instance where the fish were maintained in a tank whose water was known to contain the organism, that infection did not arise from simple ingestion, although it must be admitted that these conditions may well be changed following starvation for any length of time, and which may reduce the natural resistance of the fish to the attacks of pathogenic bacteria.

It will have been seen from the information given earlier in this thesis, that the area immediately surrounding the fishpool had previously been sprayed with a weedkilling compound. This had raised doubts as to whether the substance was toxic to fish or not and although chemical analysis failed to demonstrate the presence of the compound in the water,

the information is still of value. It is considered possible that a small quantity of the weedkiller may in fact have entered the water, and though not in amounts large enough to kill the fish outright, may have brought about a weakened state in the animals by some undetermined path. This latter hypothetical state may further have ateld upon the fish in reducing their food consumption by indirect means, allowing the pseudomonad to gain entrance to the body. In this instance, the bacterium could possibly have become established in one of the organs or systems, and from thence have spread to the remaining organs. The introduction of the organism in pure culture into the peritoneal cavity was sufficient to bring about a low degree of infection in three of the experimental fish.

Even though no ectoparasites were seen, or their lesions detected. there exists a possibility that the fish in their weakened state were subjecta to such attack, a gill parasite being of particular interest here in view of the statements of Snieszko (33), and the condition of the gills on autopsy. However, it is difficult to correlate these considerations with the fact that a large number of the total fish population of the pond were affected, and that the greater portion of these died as a result of the condition described. The isolation of the organism from the blood stream is sure evidence of its arrival thereto by some agency, though the precise nature of this agency cannot be determined.

A full consideration of the effects of chemicals upon goldfish is given by Davidow and Sabatino (10) and Davidow and Schwarzman (11). Whether the condition was developed before or after the use of the weedkiller is unknown, but circunstantial evidence points to its development following the use of the said chemicals. That the deaths did not occur immediately suggests that one does not have to suspect a chemical toxicity, and this is further supported by the bacteriological findings.

It is therefore concluded that the infection arose as a result of a combination of numerous factors. Firstly, that the chemical weedkiller may have reduced to a marked degree the feeding rate of the fish, for as has been shown by Marlborough (21), there is no reason to doubt that the fish would be actively feeding at these temperatures, and secondly that the organism was able to display its pathogenic powers following the ingestion of the chemical. This conclusion would tend to be supported by the findings of Bissett (3) that fish may succumb to infection by normally saprophytic bacteria when the environmetal conditions are subject to

change from some external agency, holding an increase in water temperature to be partly responsible. Furthermore, Jacobs (17) states that whilst serious pathogens are not likely to remain dormant, the low grade pathogens may survive for long periods, and only give rise to slight infections on the appearance of suitable conditions.

It is felt that the latter finding may explain to some extent the reasons for the outbreak described in this thesis, since the fish were apparently quite healthy before the advent of such undetermined adverse conditions.

With regard to the lack of pathogenicity under experimental conditions in the laboratory, paying particular atention to its effect upon laboratory mice, a certain loose resemblance is seen to the properties of *Serratian anolium,* whose pathogenicity to fish, amphibians, and reptiles has been investigated by Clausen and Duran-Reynals (7). These workers noted that their isolate, obtained from the iguana lizard (*Anolis equestris*) was non-virulent to the warmblooded animals they tested, no pathogenicity being seen in any of this type of animal. Similarly, an organism with properties resembling those of *Serratia anolium* was obtained from chuckawallas (*Sauromaulus varius*) by Conti and Crowley (9). This species, like *S. anolium* and the pseudomonad described herein, was pathogenic to certain poikilothermic animals, but not to guinea pigs (or white mice in the case of the pseudomonad). Of especial interest is the production of a fluorescent pigment identical to that produced by *Pseudomonas aeruginosa* and P. *fluorescens,* and for this very reason Conti and Crowley wished to include their isolate amongst the members of the genus *Pseudomonas.* In view of taxonomic difficulties raised by its flagellation however, they were content to provisionally classify their isolate as *Bacterium sauromauli.* Conversely, the *Pseudomonas reptilivora* of Caldwell and Ryerson (5) was shown to be highly pathogenic to rabbits and guinea pigs, in addition to horned lizards (*Phrynosoma solare*), Gila monsters (*Hemoderma suspectum*), and chuckawallas (*Sauromalus ater*). No satisfactory explanation can therefore be given to account for the apparent lack of pathogenic powers to white mice by the isolate under discussion, other than suggesting that healthy animals are not attacked, a fact apparently borne out by the experimental inoculation of goldfish. The increase in the number of monocytes in the mice must be emphasised, for those are the phagocytic cells of the reticulo-endothelial system, and are

nearly always encountered in bacterial infections, particularly those of the blood stream.

Thus it is felt that there exists a sufficient precedence for the provisional classification of the organism isolated from the condition described in this thesis as a "pseudomonad". This step is taken after due reference to the appropriate literature, and it must remain to be seen whether future reseach will reveal that the organism is in fact a new species, or a strain of an already existing species.

SUMMARY

The organism isolated from an oedematous and haemorrhagic condition in goldfish (*Carassius carassius* var. *auratus*) is described, and an attempt is made to trace the course of the infection by experimental studies. It appears that the organism possesses certain characteristics in common with those described for *Pseudomonas* (*Aeromonas*) *punctata,* and *P. putida,* but its precise classification is left open to doubt, and the organism is provisionally classificied as nothing more than a "pseudomonad". A detailed account of the studies effected on the pathogenicity of the organism is given, and a review of the literature available on similar diseases made.

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The author wisher to express his sincere thanks to Prof. Dr. Luis C. Verna, of this Department for his continued interest in the investigation described, and for having offered every facility to effect this study; and to his friends Drs. F. N. Ola and V. L. Orce, also of this Department, for adding their advice throughout the preparation of this thesis.

Sincere thanks are also due to Drs. S. F. Snieszko and R. R. Rucker, of the United States Department of the Interior (Fish and Wildlife Service), who both so freely gave of their experience of fish diseases caused by bacteria; and to Mr. C. van Duijn for the provision of much detailed information on the subject.

The kindness of Dr. G. J. Kane, formerly Head of the Department of Veterinary Pathology, Berkhamsted Hill Research Station, England, in giving much valuable help and advice; and to Mr. K. Tyskiewicz, Bacteriologist in the same Department, for the provision of freeze-dried cultures for detailed studies, is herein gratefully acknowledged.

RESUMEN

Se describe el organismo aislado de la carpa dorada (*Carassius carassius* var. *auratus*) con edema hemorrágico y se intenta averiguar el curso de la infección mediante un estudio experimental. Parece que el organismo posee ciertas características comunes con las del *Pseudomonas* (*Aeromonas*) *punctata* y del *P. putida*, pero su clasificación exacta es dudosa y en consecuencia, se clasifica provisionalmente como un "pseudomonádido". Se exponen detalladamente los estudios llevados a cabo acerca de la patogenicidad del organismo y, asimismo, se recoge la bibliografía sobre enfermedades similares.

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C. S. I. C.
INSTITUTO «JAIME FERRAN», DE MICROBIOLOGIA

ESTUDIO DE LOS EFECTOS QUE PRODUCE LA LUZ ULTRAVIOLETA SOBRE LAS BACTERIAS

III. Alteraciones del comportamiento fisiológico del *Mycobacterium phlei*

POR

EULALIA CABEZAS DE HERRERA (*)

Siguiendo la serie de trabajos orientados a estudiar las alteraciones fisiológicas que se producen en la célula bacteriana por efecto de la radiación ultravioleta, a continuación damos a conocer los resultados obtenidos con el *Mycobacterium phlei.*

MATERIAL Y METODOS

Como en los trabajos anteriores, hacemos todas las experiencias con cultivos sincrónicos, obtenidos por el método de Scott y Chu (3).

Estirpe

La estirpe con que trabajamos procede del Laboratorio de Microbiología de la Universidad de Amsterdam, conservada en agar-caldo a 4 °C.

Sus características las fijamos: por el comportamiento frente a 37 medios de cultivo *(cuadro 1)*, en los que el indicador que llevan los azúcares es el púrpura de bromocresol; y por sus curvas de crecimiento, cultivando la estirpe en caldo común a 37 °C, en reposo y con agitación *(figura 1)*.

(*) Agradecemos a la Fundación "Juan March" la beca concedida para la realización de este trabajo.

Las curvas las obtenemos con el fotocolorímetro de Klett-Summerson, modelo 800-3 y con los matraces especialmente diseñados para este fin (1).

Cuadro 1

Medios de cultivo	Estirpe					
	Testigo	Irradiada, min				
		5	15	30	60	90
Acetato de plomo	+	+	+	+	—	+
Adonita	—	—	—	—	—	—
Agua de peptona	—	—	—	—	—	—
Albúmina de huevo	—	—	+	—	+	—
Almidón	—	—	—	—	—	—
Arabinosa	+	+	+	+	+	+
Caldo común	+	+	+	+	+	+
Caldo con ClNa	+	+	+	+	+	+
Caldo con NO_3K al 1 %	+	+	+	+	+	+
Caldo con urea al 1 %	+	+	+	+	+	+
Celobiosa	—	—	—	—	—	—
Dextrina	—	—	—	—	—	—
Dulcita	—	—	—	—	—	—
Eritrita	—	—	—	—	—	—
Galactosa	—	—	—	—	+	—
Gelatina	—	—	—	—	—	—
Glicerina	—	—	—	—	—	—
Glicógeno	—	—	—	—	—	—
Glucosa	+	+	+	+	+	+
Inosita	—	—	—	—	—	—
Inulina	—	—	—	—	—	—
Koser	+	+	+	+	+	+
Lactosa	—	—	—	—	—	—
Leche tornasolada	—	—	—	—	—	—
Levulosa	+	+	+	+	+	+
Maltosa	—	—	—	—	—	—
Manita	+	+	+	+	+	+
Manosa	+	+	+	+	+	+
Melobiosa	—	—	—	—	—	—
Metil-glucósido	—	—	—	—	—	—
Rafinosa	—	—	—	—	—	—
Rhamnosa	—	—	—	—	—	—
Sacarosa	+	+	+	+	+	+
Sorbita	+	+	+	+	—	+
Trehalosa	+	+	+	+	+	+
Voges-Proskauer	±	±	±	±	±	—
Xilosa	±	±	±	±	±	±

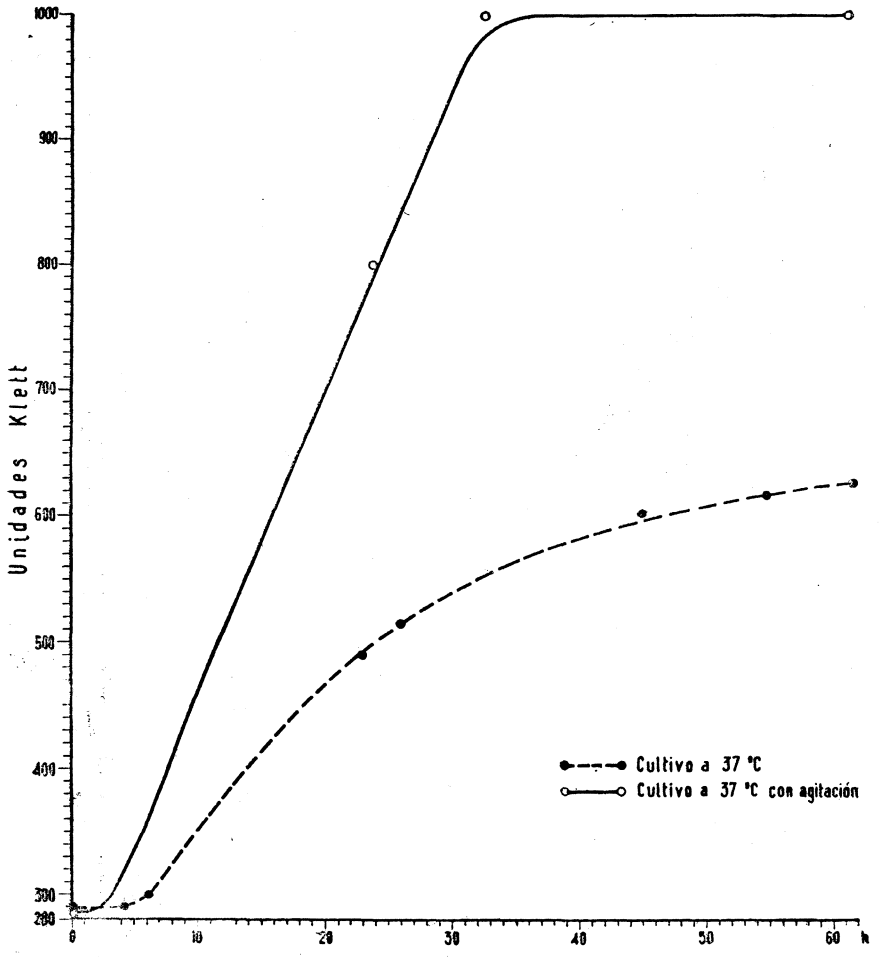

Figura 1

Preparación de las muestras

De un cultivo en plena fase logarítmica de crecimiento (veinte horas), tomamos una determinada cantidad y diluimos con caldo común fresco hasta una concentración celular de $10^8/cm^3$.

La concentración celular la determinamos por el método de recuento en placa.

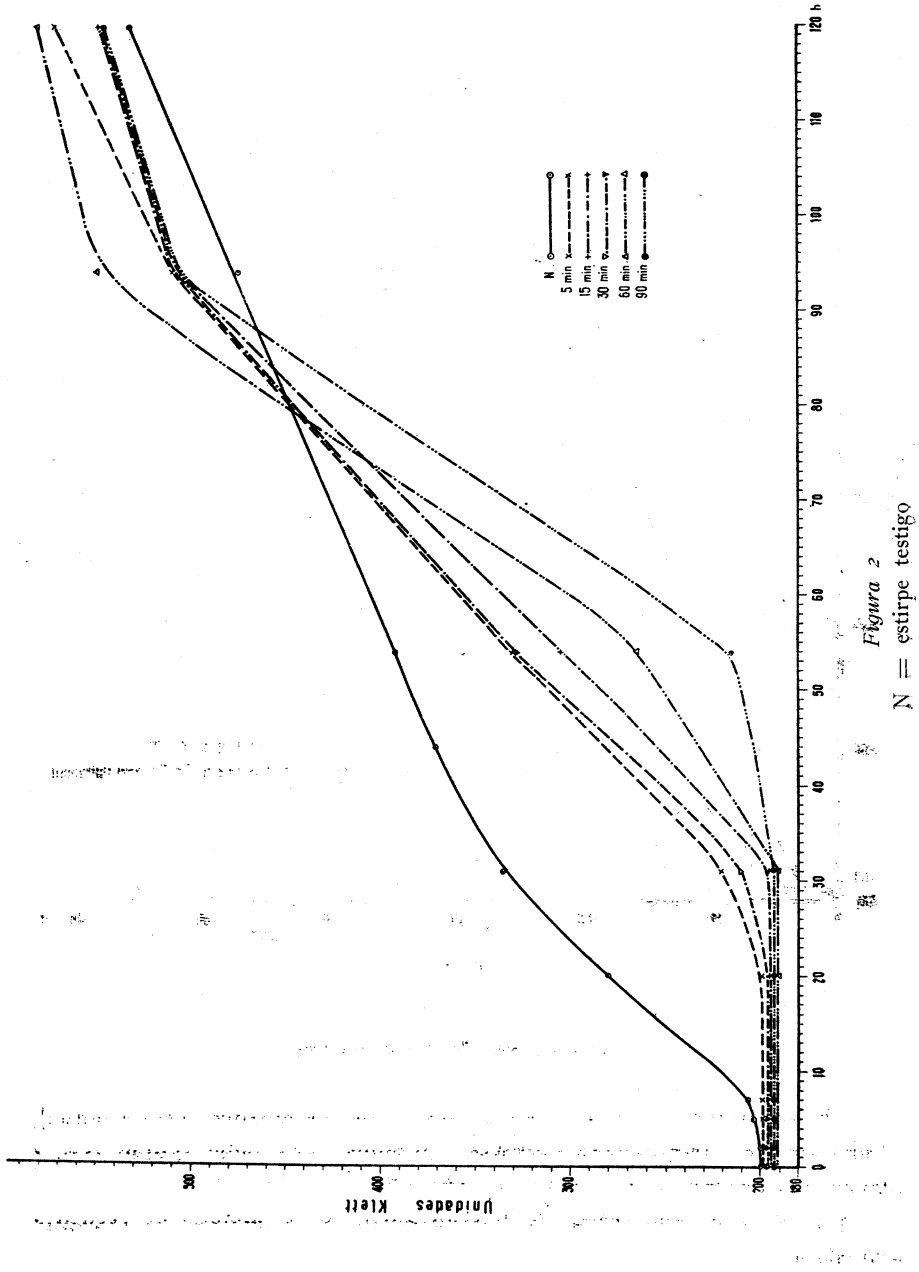

Figura 2
N = estirpe testigo

Irradiación

En cada placa Petri de 4,5 cm de diámetro, ponemos 2 cm^3 de la suspensión bacteriana de 10^8 células/cm^3.

A 14,5 cm de distancia del foco de emisión, irradiamos desde cinco a noventa minutos con 4 lámparas de mercurio Sylvania de 15 W cada una, las cuales emiten una longitud de onda de 2.537 Å.

Durante la irradiación, las muestras son constantemente agitadas con un agitador de vaiven, de 113 r/m sobre el que está colocada la cámara de luz ultravioleta. La intensidad efectiva de la luz ultravioleta a través de la muestra la calculamos por el método de Morowitz (2).

Una vez irradiada la bacteria, la sembramos en caldo común fresco para obtener las curvas de crecimiento, y en los distintos medios de cultivo, para conocer sus modificaciones fisiológicas.

En ambos casos cultivamos 37 °C.

Todas las experiencias las realizamos en cámara oscura.

RESULTADOS

En el *cuadro 1* se refleja el diferente comportamiento de la estirpe testigo y la irradiada. La estirpe irradiada, a su vez, se comporta de una manera diferente, según el tiempo que haya estado expuesta a la luz ultravioleta.

La *figura 2* nos muestra cómo las curvas de crecimiento también acusan las alteraciones producidas en la estirpe irradiada.

RESUMEN

Estudiamos las alteraciones que la luz ultravioleta produce en el comportamiento fisiológico del *Mycobacterium phlei* en cuanto a su curva de crecimiento y comportamiento frente a diferentes medios de cultivo.

SUMMARY

The influence of U. V. irradiation on the physiological activities of *Mycobacterium phlei* in different culture media and on its growth curve is studied.

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ESTUDIO DE LOS EFECTOS QUE PRODUCE LA LUZ ULTRAVIOLETA SOBRE LAS BACTERIAS

IV. Alteraciones del comportamiento fisiológico del *Staphylococcus aureus*

POR

EULALIA CABEZAS DE HERRERA (*)

En trabajos anteriores hemos estudiado las alteraciones que por efecto de la luz ultravioleta se producen en el comportamiento fisiológico de estirpes tan dispares como *Eschericia coli, Bacillus cereus* y *Mycobacterium phlei.* En el presente trabajo estudiaremos las que se produzcan en el *Staphylococcus aureus,* estirpe, a su vez, muy diferente a las anteriores.

Hemos elegido diferentes tipos de bacterias con el fin de observar si la organización específica de cada una de ellas reaccionaba de una manera propia a la acción de la radiación, o si, por el contrario, existe una respuesta común al mismo estímulo.

MATERIAL Y METODOS

Seguimos el método de Scott y Chu (3) para lo obtención de cultivos sincrónicos, con los cuales realizamos todos los experimentos.

(*) Agradecemos a la Fundación "Juan March" la beca concedida para la realización de este trabajo.

Cuadro 1

Medios de cultivo	Estirpe					
	Testigo	Irradiada, min				
		5	15	30	60	90
Acetato de plomo	+	+	+	+	+	+
Adonita	—	—	—	—	—	—
Agua de peptona	—	—	—	—	—	—
Albúmina de huevo	+	—	—	—	—	—
Almidón	+	+	+	—	+	—
Arabinosa	+	—	—	—	—	+
Caldo común	+	+	+	+	+	+
Caldo con ClNa	+	+	+	—	—	—
Caldo con NO_3K al 1 %	—	—	—	—	—	—
	(*)	(*)	(*)	(*)	(*)	(*)
Caldo con urea al 1 %	+	+	+	+	+	+
Celobiosa	—	—	—	—	—	—
Dextrina	+	+	+	+	+	+
Dulcita	—	—	—	—	—	—
Eritrita	—	—	—	—	—	—
Galactosa	+	+	+	+	+	—
Gelatina	+	+	+	+	+	+
Glicerina	+	+	+	—	—	—
Glicógeno	—	—	—	—	—	—
Glucosa	+	+	+	+	+	+
Inosita	—	—	—	—	—	—
Inulina	—	—	—	—	—	—
Koser	±	±	±	±	±	±
Lactosa	+	+	+	+	+	+
Leche tornasolada	+	+	+	+	+	—
Levulosa	—	—	—	—	—	—
Maltosa	+	+	+	+	+	+
Manita	+	+	+	+	+	+
Manosa	+	+	+	+	+	+
Melobiosa	—	—	—	—	—	—
Metil-glucósido	—	—	—	—	—	—
Rafinosa	—	—	—	—	—	—
Rhamnosa	—	—	—	—	—	—
Sacarosa	+	+	+	+	—	+
Sorbita	—	—	—	—	—	—
Trehalosa	—	—	—	—	—	—
Voges-Proskauer	—	—	—	—	—	—
Xilosa	+	+	+	+	+	+

(*) Crece, pero no reduce nitratos.

Estirpe

Staphylococcus aureus, procedente de la Colección Nacional de Cultivos tipo, de Londres.

Conservamos la estirpe sobre agar-caldo a 4 °C.

Fijamos sus características: estudiando su comportamiento frente a 37 medios de cultivo *(cuadro 1),* en los que los azúcares llevan como indi-

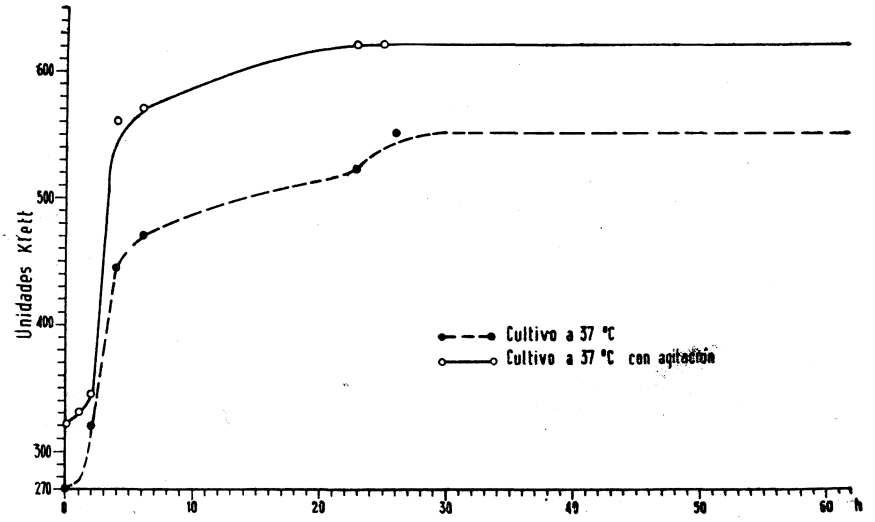

Figura 1

cador púrpura de bromocresol; y obteniendo sus curvas de crecimiento en caldo común a 37 °C, en reposo y con agitación *(figura 1).* Las curvas las obtenemos con el fotocolorímetro de Klett-Summerson, modelo 800-3 y con los matraces diseñados para este fin (1).

Preparación de las muestras

Tomamos una determinada cantidad de un cultivo en fase logarítmica de crecimiento (diecisiete horas) y diluimos con caldo común fresco hasta una concentración celular de $10^9/cm^3$.

Irradiación

En placas Petri de 4,5 cm de diámetro ponemos 2 cm^3 de la suspensión bacteriana de 10^9 células/cm^3.

Durante la irradiación, las muestras son constantemente agitadas por un agitador de vaivén de 113 r/m sobre el que está instalada la cámara.

Disponemos de una cámara con paredes de aluminio con 4 lámparas de mercurio Sylvania de 15 W, que emiten una longitud de onda de 2.537 Å.

Las placas las colocamos sobre una bandeja a 14,5 cm del foco de emisión, e irradiamos durante cinco, quince, treinta, sesenta y noventa minutos.

La intensidad efectiva proporcionada a través de la muestra la calculamos por el Método de Morowitz (2).

Una vez irradiada la bacteria, sembramos en los 37 medios de cultivo con que fijamos sus características y en caldo común en los matraces ya indicados, para obtener la curva de crecimiento, para comparando con la estirpe testigo, estudiar las alteraciones sufridas por efecto de la luz ultravioleta. Cultivamos a 37 °C.

Todas las operaciones las realizamos en cámara oscura.

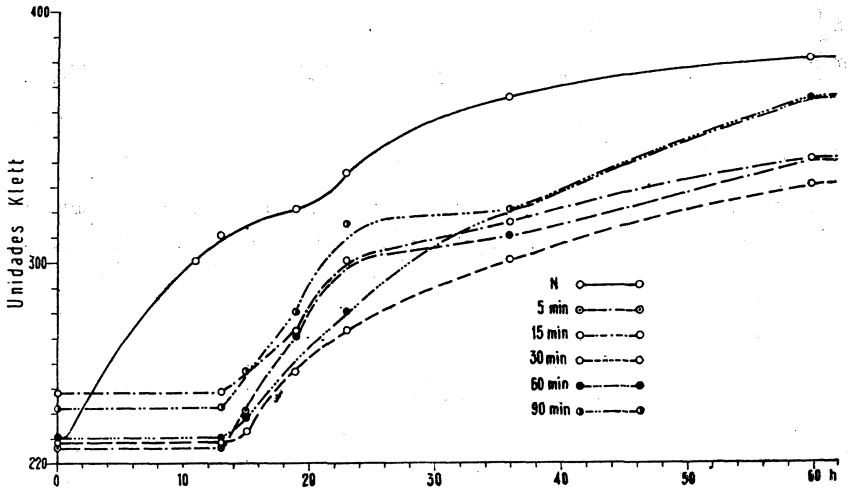

Figura 2

N = estirpe testigo

RESULTADOS

En el *cuadro 1* están reflejadas las alteraciones producidas en la estirpe por efecto de la radiación, en el diferente comportamiento de la estirpe irradiada frente a los medios de cultivo ensayados.

La *figura 2* nos muestra cómo las curvas de crecimiento tienen una fase de adaptación mayor en la estirpe irradiada.

RESUMEN

Estudiamos las alteraciones producidas por efecto de la luz ultravioleta en una estirpe de *Staphylococcus aureus* en cuanto a su comportamiento frente a 37 medios de cultivo diferentes, y a su curva de crecimiento.

SUMMARY

The influence of U. V. irradiation on the physiological activities of *Staphylococcus aureus* in different culture media and on its growth is studied.

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C. S. I. C.
INSTITUTO «JAIME FERRAN», DE MICROBIOLOGIA

ESTUDIO DE LOS EFECTOS QUE PRODUCE LA LUZ ULTRAVIOLETA SOBRE LAS BACTERIAS

V. Alteración del metabolismo de los ácidos nucleicos del *Mycobacterium phlei*

POR

EULALIA CABEZAS DE HERRERA (*) y PILAR AZNAR

Aunque se han hecho muchas investigaciones sobre el contenido de los ácidos nucleicos en las bacterias, no se han realizado de manera sistemática hasta los estudios de Cohen (5) y Malgren (16).

Los resultados de Malgren muestran que el contenido total de nucleótidos de las células individuales de un cultivo cambia durante el crecimiento del mismo, llegando al máximo cerca del final de la fase latente o estacionaria.

Morse y Carter (19) estudiaron la síntesis de los ácidos nucleicos durante las primeras fases del ciclo de crecimiento de la *Escherichia coli*, estirpes B y Br.

Hotckiss (10), trabajando con cultivos sincronizados, estudia la transformación del ADN durante el período inmediatamente anterior a la división del neumococo.

Lark y Maaloe (15) estudiaron la relación entre la síntesis de los ácidos nucleicos y la división celular en la *Salmonella typhimurium*.

Maruyama (17) mostró el aspecto dinámico de los cambios bioquímicos ocurridos durante el crecimiento sincrónico de la *E. coli*.

Entre los numerosos trabajos relativos al metabolismo de los ácidos nucleicos, puede seleccionarse un grupo de ellos (1-2, 6, 8, 11, 20, 23-26).

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El descubrimiento de Kelner (14), de que la luz ultravioleta bloquea específicamente la síntesis del ADN en la *E. coli,* confirmado después por otros autores (9, 12-13), ha subrayado el papel que juega el metabolismo de posirradiación del ADN, en el efecto de la radiación sobre la célula. La recuperación de esta inhibición por la luz (14), y la demostración por Rupert y cols. (21) de la fotorreactivación *in vitro* del *Haemophilus influenzae* inactivado por luz ultravioleta, parece probar que, al menos, la inactivación de la porción reactivable por efecto de la luz, puede estar relacionada con el trastorno en el metabolismo del ADN.

En el presente trabajo estudiaremos, primero, las alteraciones que produce la luz ultravioleta en el metabolismo de los ácidos nucleicos de la *E. coli* (estirpe que ha sido estudiada por Kanazir y Errera (13) y Kelner (14) para comprobar el procedimiento; después estudiaremos las que se producen en el metabolismo de los ácidos nucleicos del *M. phlei,* estirpe que no ha sido estudiada hasta el momento.

MATERIAL Y METODOS

Estirpe

La estirpe de *E. coli* utilizada en este experimento es la K_{12}, procedente del Instituto Pasteur, cuyas características ya detallamos (3); la de *M. phlei* procede del Laboratorio de Microbiología de la Universidad de Amsterdam, con características también descritas (4). Ambas estirpes se conservan en agar-caldo a 4 °C.

Para las experiencias, cultivamos en caldo común.

Preparación de las muestras

Cultivamos en caldo común durante la noche, cultivos sincrónicos de *E. coli* (diecisiete horas) y *M. phlei* (veinte horas). Con estos cultivos en fase logarítmica de crecimiento, inoculamos y cultivamos a 37 °C.

A las dos, cuatro, cinco, seis, ocho, doce, veinticuatro y cuarenta y ocho horas de cultivo enfriamos a 5 °C, centrifugamos, lavamos con agua destilada a 5 °C, recogemos el sedimento y lo desecamos en baño María.

Irradiación

Irradiamos con 4 lámparas de mercurio Sylvania de 15 W cada una. En una bandeja que está a 14,5 cm del foco de emisión, disponemos placas Petri de 4,5 cm de diámetro. En cada placa ponemos 2 cm^3 de la suspensión bacteriana.

Irradiamos durante treinta y sesenta minutos. Durante la irradiación la cámara está movida por un agitador de vaivén de 113 r/m.

Después de irradiadas las bacterias, las sembramos en caldo común fresco y cultivamos en oscuridad durante cuatro, cinco, seis, ocho, doce, veinticuatro y cuarenta y ocho horas.

Todas estas operaciones las realizamos en cámara oscura. Transcurrido el tiempo de cultivo, enfriamos a 5 °C, centrifugamos, lavamos con agua destilada a 5 °C, recogemos el sedimento y desecamos en el baño María.

Valoración de los ácidos nucleicos

La valoración de los ácidos nucleicos la efectuamos por el método de Schmidt y Thannhauser (22); la del fósforo por el de Fiske y Subbasow (7).

Pueden ejercer alguna influencia en el desarrollo de la coloración, el pH y ciertos componentes de los extractos, por ello no se puede emplear una curva patrón para todas las determinaciones; nosotros operamos según Melnick y Field (18), añadiendo a una parte alícuota del líquido que se analiza, una proporción exactamente conocida de fósforo tomado de una solución patrón. De esta manera hallamos el valor del coeficiente de extinción debido al derivado coloreado procedente del fósforo, en cada caso particular.

Empleamos el fotómetro de Pulfrich-Zeiss eligiendo el filtro más adecuado, que en nuestro caso es el S-75, según puede verse en la *figura 1*.

Comprobamos el cumplimiento de la ley de Beer haciendo colorimetrías con soluciones patrones de ácidos ribonucleico y desoxirribonucleico, con concentraciones que varían entre 0,2-0,8 mg. En las *figuras 2-3* puede verse el satisfactorio ajuste de las rectas obtenidas entre estos límites de concentración.

Hacemos determinación de materia seca de las muestras para referir los resultados a tanto por ciento de la misma.

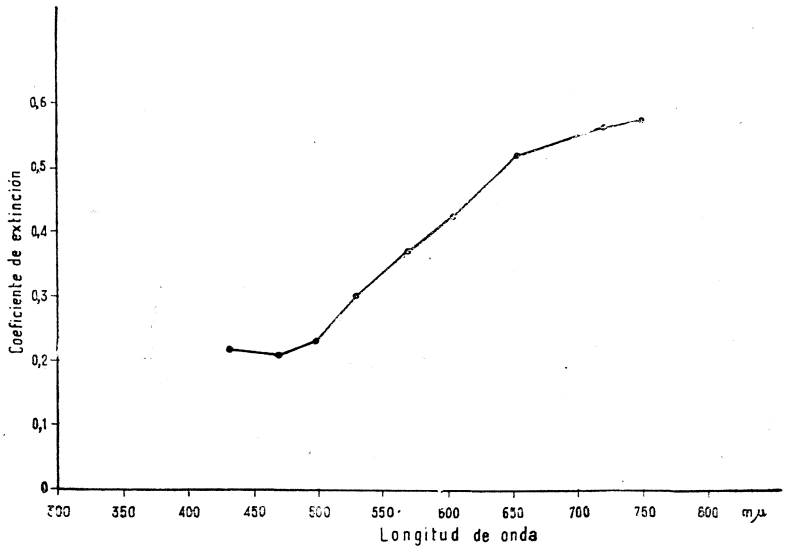

Figura 1. Coeficiente de extinción de los filtros

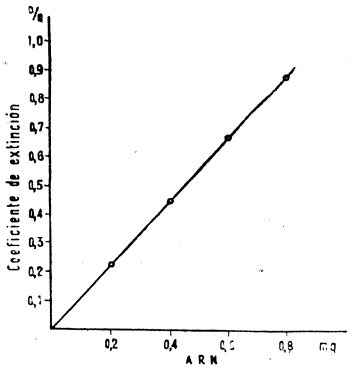

Figura 2. Comprobación de la ley de Beer en una solución patrón de ARN

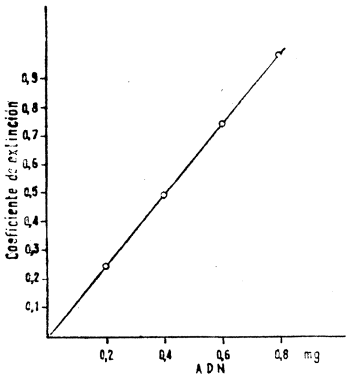

Figura 3. Comprobación de la ley de Beer en una solución patrón de ADN

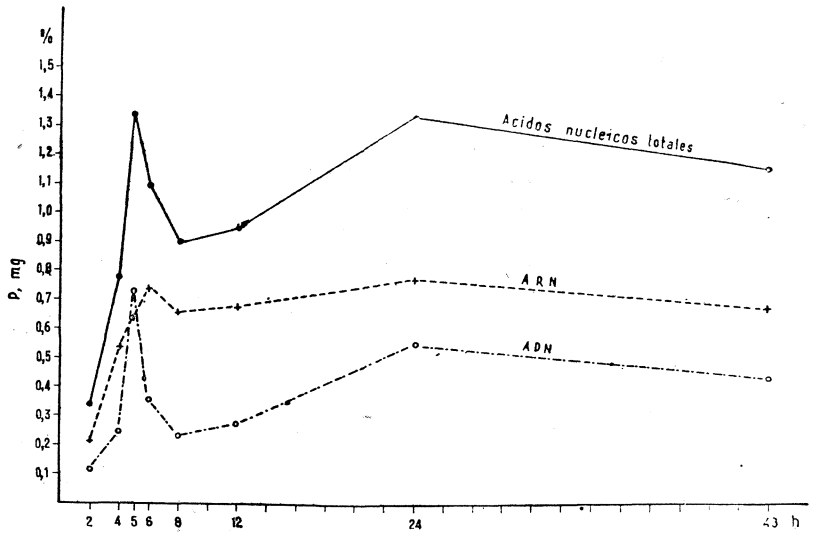

Figura 4. Metabolismo de los ácidos nucleicos de E. coli, *estirpe testigo*

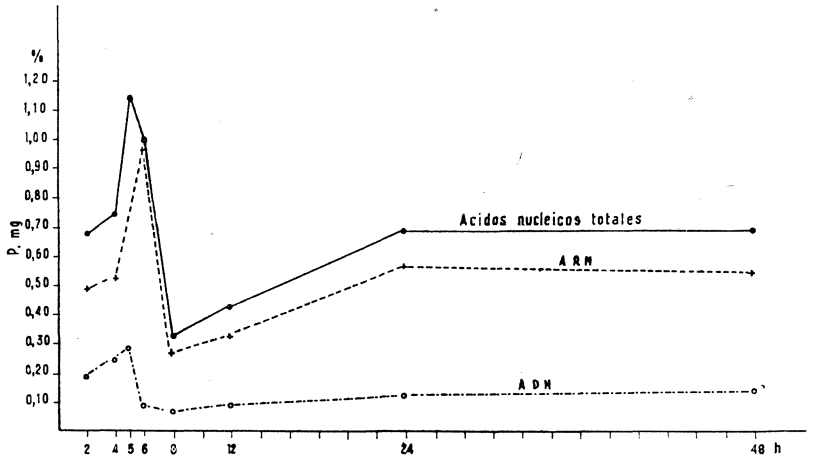

Figura 5. Metabolismo de los ácidos nucleicos de M. phlei, *estirpe testigo*

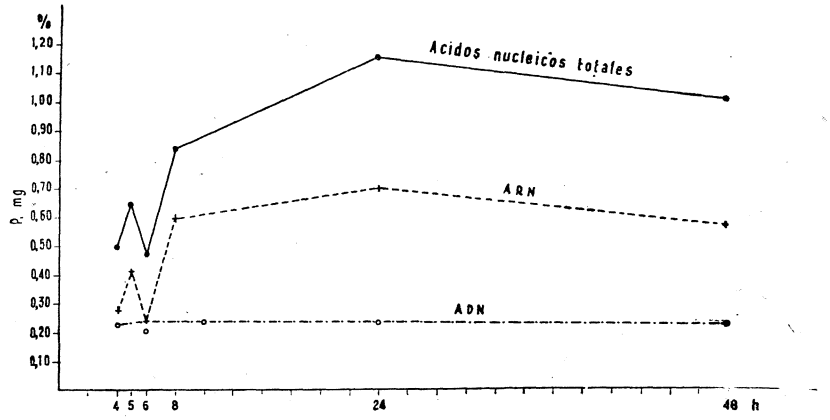

Figura 6. Metabolismo de los ácidos nucleicos de E. coli *irradiada durante treinta minutos*

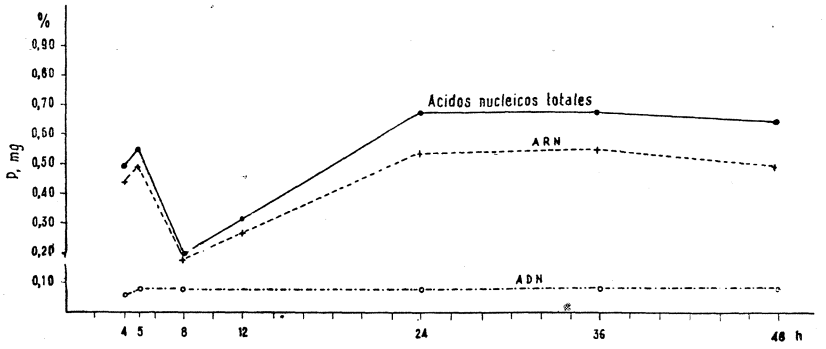

Figura 7. Metabolismo de los ácidos nucleicos de M. phlei *irradiado durante sesenta minutos*

RESULTADOS

Las *figuras 4-5* nos muestran el metabolismo de los ácidos nucleicos de las estirpes testigos. Las *figuras 6-7* nos muestran el metabolismo de los ácidos nucleicos de las estirpes irradiadas.

DISCUSION

En los resultados obtenidos, podemos observar que la acción de la luz ultravioleta también bloquea la síntesis del ADN en el *M. phlei.*

Esto confirma los resultados citados anteriormente (13-14) y añade un dato más a la tesis de que son los ácidos nucleicos los que inicialmente absorben la radiación y es lo que causa la inhibición de la división de la célula.

RESUMEN

Estudiamos el metabolismo de los ácidos nucleicos de las estirpes testigos e irradiada de *Mycobacterium phlei,* observando el bloqueo de la síntesis del ácido desoxirribonucleico en la estirpe irradiada.

SUMMARY

The metabolismo of nucleic acids of normal and U. V. *Mycobacterium phlei* strains was studied.

The synthesis of deoxiribonucleic acid in the irradiated strain was inhibited.

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