ERO-9-72

# US ARMY RESEARCH and DEVELOPMENT GROUP (EUROPE)
## Box 15, FPO New York 09510

AD 745414

MICROBIOLOGICAL RESEARCH IN SPAIN:
NOTES ON FIVE LABORATORIES

by

J.B. BATEMAN
June 1972

DDC
JUL 26 1972
C

## EUROPEAN RESEARCH OFFICE
## Room 604, Keysign House
## 429 Oxford Street
## London W.1. ENGLAND

Reproduced by
NATIONAL TECHNICAL
INFORMATION SERVICE
U S Department of Commerce
Springfield VA 22151

29

TECHNICAL REPORT

# MICROBIOLOGICAL RESEARCH IN SPAIN: NOTES ON FIVE LABORATORIES

by

J.B. BATEMAN

CONTENTS


| | | Page |
|---|---|---|
| I. | INTRODUCTION | 2 |
| II. | THE CENTRO DE INVESTIGACIONES BIOLOGICAS, MADRID | 2 |
| | a. Introduction | 2 |
| | b. Instituto "Jaime Ferrán" | 3 |
| | c. Instituto de Biología Celular | 7 |
| III. | UNIVERSIDAD DE BARCELONA | 9 |
| | a. Microbiology in the Faculty of Medicine | 9 |
| | b. Microbiology in the Faculty of Science | 11 |
| IV. | THE UNIVERSIDAD AUTÓNOMA DE BARCELONA, DEPARTMENT OF MICROBIOLOGY | 18 |
| V. | SUMMARY AND COMMENT | 19 |
| VI. | REFERENCES | 20 |
| VII. | DISTRIBUTION | 23 |
| VIII. | ANNEX: The Seven Research Institutes of the Centro de Investigaciones Biologicas, Madrid | 25 |

## I. INTRODUCTION

The choice of laboratories of microbiology to visit in Spain was made in several ways. The "Memoria" of the Centro de Investigaciones Biologicas (1, 2) are a valuable guide to the research interests of the center, and several of the seven member institutes touch on microbiology in one form or another. Correspondence had already been pursued in connection with one scientist who had submitted a research proposal. There was also the useful ONR (London) report of Dr. Hottle (3) and, finally, the on-the-spot recommendations of other scientists both in Madrid and Barcelona. No suggestion was made in these conversations that any particularly important work in microbiology is being done elsewhere in Spain although, for reasons of professional loyalty perhaps, the possibility was not actually denied.

It is not intended that this report shall duplicate that of Dr. Hottle. I did talk with some of the same people, but the questions I asked led sometimes to discussion of different topics from those mentioned in his paper and we may have received somewhat different impressions.

## II. THE CENTRO DE INVESTIGACIONES BIOLOGICAS, MADRID

### a. Introduction

In the "Memoria" (1, 2) it is stated that under the law of the Senior Council for Scientific Investigations, the Ramón y Cajal Foundation is assigned responsibility for investigations in medical sciences and animal biology, and for establishment of an appropriate research center. Accordingly, seven institutes were founded in 1969, with a 1970 strength of 237 technical personnel, and a budget of nearly 89 million pesetas or about $14 million, mostly from the Ministry of Education and Science. They occupy a large brick building designed to fit quite neatly into the acute angle formed by the intersection of Velazquez and Joaquín Costa Streets not far from the center of the city. The satisfactory external shape is not reflected internally by any features that would suggest intelligent planning for research laboratories. Space available to an investigator is measured by the number of windows: dirty windows, it may be added, because the architect, seized with enthusiasm for double glazing after a visit to Sweden, but faced with a frugal budget, modified the design so as to make cleaning a major operation. In a building with perhaps 300 windows, at a rough estimate, it takes two men an entire day to clean two of them.

The seven institutes, with their several departments and sections, are listed in the Annex. A little more information about the Institute of Genetics and Anthropology and a discussion of the work of the Section on Human Genetics will be found in another report (4).

b. Instituto "Jaime Ferrán"

This Institute, headed by Professor Lorenzo Vilas López, is really an institute of microbiology, with departments of Virology, Bacteriology, General Microbiology and Protozoology; Professor Vilas acts also as head of General Microbiology. The various sections are listed in the Annex.

After describing the organization of the Institute and hearing something about the functions of the European Research Office, Professor Vilas introduced the persons whose interests might, in his judgement, coincide with those of ERO: these were Dr. Miguel Rubio Huertos, head of the Department of Virology and its section on plant viruses, Dr. Angel Procopio García Gancedo, head of the animal virus section, and Mr. Antonio Portolés Alonso, head of the section on the biology of bacterial infections, Department of Bacteriology. This resulted in a discussion of a few particular subjects rather than a run-down of all the activities of the Institute, which will be found listed in the "Memoria" (1, 2).

Immunodepressive effects of Pseudomonas aeruginosa metabolites: Dr. Portolés has been interested in the antibiotic resistance of Pseudomonas and of clinical infections with the organism since about 1960. In 1963 he published the results of a competently executed but rather pedestrian study of the inhibitory effect of 15 antibiotics singly, in their 101 binary mixtures (certain mixtures of tetracyclines having been omitted), and in selected trivalent combinations, on 80 strains of P. aeruginosa isolated from clinical infections (5). Following casual observation and similar reports in the literature, this led to examination of a possible relationship between the effects of antibiotics and the nature and quantity of the pigments produced (6), using the techniques of Sephadex filtration and elution of the extracellular liquids, automatic fraction collection, and infrared spectroscopy on KBr pellets. By these means the various chromogens could be distinguished--pyocyanins, oxyphenoxazones and phenazines, in previously known and in several unknown forms. These varied greatly in nature and proportions with the concentrations and nature of the different antibiotics.

The foregoing investigations evidently provided a good technical background for the current work on the biological and clinical significance of the soluble extracellular substances produced by Pseudomonas, an immunosuppressive effect having been suspected by others because of the low levels of specific antibodies often found in human Pseudomonas infections. Accordingly, extracellular polysaccharide, capsular substance and fractionated pigments were prepared and examined for their effects on the immune responses of rabbits and mice to formalinized vaccines prepared from Escherichia coli and Staphylococcus aureus (7). The tests made included (a) measurements of bacterial agglutinin response to challenge with the vaccines, (b) examination of respiratory inhibition of bacteria by the immune sera, (c) measurement of the phagocytic activity of peritoneal cells from immunized mice when incubated in vitro with the extracellular Pseudomonas substances and (d) the lymphocytic response and phagocytic activity of peritoneal macrophages from mice injected with the test substances and subsequently challenged by intraperitoneal injection of live S. aureus.

The humoral responses appeared both by (a) the agglutination and by (b) the respirometric tests to be strongly inhibited by the metabolites tested, but there were discrepancies: the depression of respiratory inhibition was seen to be about the same for all these substances, while the suppression of agglutination was a good deal more marked with the polysaccharides than with the pigments. The cellular responses likewise seemed to be strongly modified by the Pseudomonas metabolites. The production of peritoneal cells in response to infection with S. aureus was inhibited in both normal and vaccinated animals, as was their phagocytic index. In the in vitro tests the phagocytic activity of cells from vaccinated animals was inhibited by contact with the metabolites to values below those found in normal unvaccinated controls.

Thus the evidence for immunosuppresssive effects of both polysaccharide and pigments at humoral and cellular levels seems quite good; further, the preparations were quite toxic (mouse LD 50s: polysaccharide 7.5, phenazines 25, phenoxazone 12.5 mg/kg), though whether as a direct consequence of an immunosuppressive or other mechanism is not clear. In any case I cannot grasp the need for the author's conclusion that there are

> "two types of immunosuppressive action: ...an inert material inhibiting the normal phagocytic activity (polysaccharides) or as a cytotoxic substance acting against the white cells by interfering with their normal metabolism (chromogens)." (7)

These conclusions do not seem to follow from the data presented.

It is reported in the "Memoria" (ref 2, p. 60) that an account of the Pseudomonas projects directed by Dr. Portolés' section on the Biology of Infectious Bacteria has received two national awards, amounting to 60,000 pesetas ($858) from the firms Jorba and Pfizer. Dr. Portolés is continuing this work in an effort to identify the subcellular targets for cell wall endotoxin attack. He is now using the lysozyme--antilysozyme system for convenience as a replacement for the bacterial vaccines and is hoping to prepare $^{32}P$ and $^{56}Fe$-labelled endotoxin. A preliminary study of the purification of the protein-lipopolysaccharide complex has appeared from Dr. Rubio's department (8).

Research on African swine fever: Work on this important virus disease, which in Spain is comparable in its potential effects to foot-and-mouth disease in Britain, is being done under the direction of Dr. Gancedo in Dr. Rubio's department and with the latter's cooperation in electron microscopy. The major approach is in the search for an interfering virus or an infective non-pathogen, with the support of the necessary techniques for cultivation and maintenance of this hexagonal DNA virus, for assay by cell culture and hemadsorption techniques, and differentiation from related virus diseases of swine. My attention was directed to two papers, one dealing with virus identification and the other with the search for interfering viruses.

The first paper (9) introduces the use of pig kidney cells or established cell lines instead of the leucocyte cultures used by other investigators. These cells when infected with African swine fever virus (ASFV) adsorb erythrocytes, but the value of this in identifying ASFV is decreased by the fact that there is also some non-specific (control) hemadsorption. Some modest progress was made in establishing ways of minimizing this non-specific component.

The second paper (10) examines the effect of infection with ASFV upon subsequent infection with a series of viruses: herpes simplex, vaccinia, eastern and western equine encephalitis, Newcastle disease, Sendai virus and porcine enterovirus PQ151. The criterion used was the occurrence, or not, of cytopathological effects of the challenging virus, and the infected cells were primary porcine kidney cell cultures less than two weeks of age. Only in the case of herpes simplex virus (HSV) was any significant interference produced by prior infection with ASFV. This interaction was confirmed in three ways. First, the HSV in the cell suspensions previously infected with ASFV was titrated in chick chorioallantoic membrane (after an unstated interval, presumably, for growth of the HSV). The number of lesions was less by a factor of four than in uninfected controls. Second, the chick embryo was used instead of the porcine kidney cells and was infected with ASFV via the vitelline sac in eight-day embryos or the allantoic sac in ten-day embryos, followed at 12 days of age by the HSV and two days later by excision of the chorioallantoic membrane and counting of lesions. The interference produced by ASFV was more pronounced when infection occurred at the earlier stage (eight-day embryo). Third, the need for a sufficient interval between the two infections was shown in more detail by infecting with HSV 3,4,5,6 and 7 days after ASFV. The HSV titer in the chorioallantoic membrane decreased smoothly by a factor of 3. This looks significant, but the results were reported graphically without any data for the control group.

The results lead Dr. García to the conclusion that the African swine fever virus can be included in the same taxonomic group as herpes simplex. The program continues with preparations for the titration of interferon in order to determine whether the observed interference can be wholly or partially independent of the induction of interferon. A brief statement in the 1969 "Memoria" (ref 1, p. 67) refers to the finding that ASFV can induce production of interferon active against both HSV and Sendai virus.

The work of the animal virus section also includes a more extensive study of the herpes virus and an attempt to find mutants. Antiviral substances are also being examined with herpes, vaccinia, and Sindbis viruses as the targets (1, page 67).

Non-reverting L forms of pathogenic bacteria: Dr. Rubio referred to the work of Dr. M. Santaolalla Cerezo whose special interest has been in the lipopolysaccharides of

bacterial cell walls and in the L forms. Dr. Rubio stated that by a long process of selection Santaolalla had been able to obtain L forms of Bacillus tetani and Agrobacterium tumefaciens which revert neither in culture nor during infection and that the infections produce the disease characteristic of the parent microorganism. These L forms also support the uptake and replication of bacteriophages.

The work on B. tetani seems not to have been published; Dr. Santaolalla has, however, spoken at several meetings, including the X International Congress of Microbiology in Mexico City, on the ultrastructure of L forms of A. tumefaciens, their relationship to the mycoplasmas, and their ability to produce plant tumors without reversion. Dr. Ramona Beltrá y Martinez de Velasco has followed this with a demonstration of tumor production by the aseptic isolated nucleic acids of phytopathogenic bacteria both in bean stems and in carrot-disc cultures (11). Since the tumors thus produced seem to satisfy accepted criteria of "authenticity" as laid down by A. Braun in 1959, Dr. Beltrá seems to have disproved the contention that living cells of A. tumefaciens are essential for their formation.

Ultrastructure of viruses and virus-infected plants: Dr. Rubio, who worked in the University of California at Berkeley a few years ago, is primarily interested in the nature and ultrastructure of infected plants. Two papers mentioned here are easily accessible; they draw attention to the presence of quasi-crystalline inclusions in plants infected with turnip yellow mosaic (12) and Petunia ring-spot (13) viruses, following upon Robley Williams' discovery in 1953 of crystalline inclusions of tobacco mosaic virus and the extraction of these inclusions from infected cells by Rubio himself. Of special interest is the occurrence of tubular crystals of circular cross section probably formed by one mode of packing of the icosahedral virus particles.

### c. Instituto de Biología Celular

This Institute, headed by Dr. David Vazquez Martínez, has the Departments of Biochemistry (also headed by Dr. Vazquez), of Cytology and of Cell Chemistry housed in the main building in Madrid, and, in addition, the Department of Microbiology at the University of Salamanca and the Department of Morphology and Physiology at the University of Sevilla. The numerous research themes are listed in the "Memoria" (1,2) and can be classified roughly as follows:

| | | |
|---|---|---|
| Biochemistry | : | Mode of action of antibiotics<br>Ribosomes<br>Cell membranes: isolation and reconstitution |
| Cytology | : | Fine structure of plant cells and cytology of cell division<br>Human cytogenetics |
| Cell Chemistry | : | Glycolysis and gluconeogenesis in microorganisms and their genetic control<br>Microbial degradation of hydrocarbons |
| Microbiology | : | Mycology: lytic enzymes, protoplasts, invertase excretion, and cell structure of fungi |
| Morphology and Physiology | : | Fifteen projects including enzymology of Chlorella, chromosome replication in E. coli Hfr, carotenoid synthesis in Phycomyces. |

Mode of action of antibiotics: Without prejudging the relative merits of the various projects and the progress made, the Department of Biochemistry seems to be the most active and productive under Dr. Vazquez, perhaps because the effort is less diffuse than those of the others.

The discussion with Dr. Vazquez was mainly concerned with matters arising from a preliminary contract proposal submitted by him to the European Research Office some time ago and evaluated informally by Dr. Fred E. Hahn of Walter Reed Army Institute of Research. Dr. Hahn recognized the merits of the proposal while offering certain suggestions by which optimum scientific feasibility might be reconciled with some degree of accommodation towards the Army's interest in antibiotic resistant pathogens. A testimony to the value and reputation of Vazquez' work can be seen in the plan of one of Dr. Hahn's colleagues to spend a year in that laboratory. Unfortunately, aside from other factors, lack of space prevented this from being realized. I saw for myself the overcrowded conditions in the laboratory despite a slight recent expansion and conditions are not likely to become tolerable for a visiting scientist until

the laboratory moves to a new building now under construction. This may not take place until 1974, when Dr. Severo Ochoa is expected to move permanently from New York to Madrid.

The technical content of the Vazquez proposal will not be discussed here since it is subject to modification. It is sufficient to say that it deals with that class of antibiotics that inhibit protein synthesis, and particularly with the enzyme, peptidyl transferase, that catalyses peptide bond formation by transfer of a peptidyl group from transfer-RNA to the aminoacyl-transfer-RNA of the growing polypeptide chain attached to the 70S ribosome characteristic of procaryotic organisms. The methods used in localization of antibiotic action within the protein synthesizing system have been reviewed by Vazquez et al (14) while Vazquez (15) has written a useful chapter on this and other modes of action of antimicrobial antibiotics.

## III. UNIVERSIDAD DE BARCELONA

### a. Microbiology in the Faculty of Medicine

The Department of Microbiology is headed by Professor Agustin Pumarola Busquets who, while coping with routine responsibilities in the areas of epidemiology, public health, microbiology and parasitology, and with a heavy teaching schedule, is able to run very effectively a research laboratory in which several epidemiological projects are being carried out. These have to do with leptospirosis, respiratory viruses, arboviruses and enteroviruses. Without immediate access to his publications, I can only report briefly my conversation with Professor Pumarola.

Leptospirosis (Weil's Disease): The disease is of concern in Spain both in human beings and in pigs. Infections in man have been associated with migratory workers in the rice fields who start the season in the south and work their way northwards as the harvest date advances. Since the first extensive epidemiological study in 1947, the following measures have been taken, or attempted, to control the infection:

1. Vaccination: The narrow spectrum of only three serotypes in Spain (in contrast to Italy where there are about 10) has made possible preparation of a good precipitated vaccine. It is particularly useful and effective because it can be administered exactly one month in advance of the harvest date when infection

will occur. All workers who have been in the fields for more than five years have some degree of acquired immunity.

2. Chemotherapy: Penicillin is very effective if given for the first six to 10 days of the harvest. In other countries tetracyclines have given better results. Administration of penicillin produces shock, of diagnostic value, by release of toxin from the spirochaetes. Shock is severe but transitory, with complete recovery in 24 hours.

3. Eradication of rats: This has met with little success.

4. Treatment of fields with calcium cyanamide to produce a low pH unfavorable to the survival of Leptospira. A transitory benefit.

5. Mechanical harvesting.

6. Drainage: Replacement of rice paddies where possible by orchards. This is being pursued in the provinces of Valencia, Alicante and other parts of Andalusia, but will have some impact on the economy because of the importance of rice as a crop for exportation. The results have been good and leptospirosis is disappearing, for example, from the Ebro delta in Tarragona.

The infection of pigs with Leptospira pomona introduced from France is of economic importance, causing abortion in 80% of the cases.

Arthropod-borne virus infections: Evidence for Tahnya virus infections is said to have been found in Spain following the epizootic occurrence in the Rhône delta. No other arboviruses are known; ticks are plentiful but no tick-borne disease has been identified. Boutonneuse fever occurred before the Civil War but is now unknown.

Respiratory virus infections: Pumarola maintains the WHO influenza reference center. Results of a survey in tropical areas have been sent to the Communicable Disease Center in Atlanta. Similar surveys of influenza and related respiratory viruses are now being done in children and in out-patients.

Enteric viruses: Isolates of a number of enteric viruses from infants were obtained and sent a year ago to Atlanta for examination, but without any response to date.

b. Microbiology in the Faculty of Science

The Department of Microbiology is housed under the roof of the large, labyrinthine, inelegant but spacious building of the Faculty of Science within easy walking distance of the Ramblas. I was told that I could not see the head, Professor Ramón Parés Farras until an inconveniently late hour despite a 5 o'clock appointment, but a colleague, Dr. J. Sancho, was kind enough to explain the program, with frequent disclaimers of any authority to interpret Professor Parés' views.

The general impression one had was that of an enthusiastic and capable group of professors and students engaged in challenging problems but handicapped by lack of modern equipment.

According to Dr. Sancho, the two principal lines of interest are in microbial taxonomy and genetics.

Microbial taxonomy: Numerical taxonomy is being combined with biochemical taxonomy and serological typing. A small IBM 1130 computer is available in the laboratory; they also have access to the faculty IBM 360 machine, although this is too small, and the possibility of using the City computer housed in the Town Hall. Experimental work is being done, including the recording of thermal transition ("melting") curves of DNA, using a painstaking manual procedure for lack of money.

Microbial genetics: Episomal information transfer: The main line of work during the past ten years, published in a series of papers in the Catalan and Spanish languages, has been the study of an organism with a particularly labile form of DNA from the point of view of episomal transfer of characteristics to other organisms. An illustration was given in Hottle's paper (3) in the induction of the ability to secrete glutamic acid in strains which normally do not do so. According to Sancho, Parés considers this to be an example of a general phenomenon which will be found to account, in part, for the complexities encountered in the classification of microorganisms. He asserted, rather cautiously, that the mechanism is not necessarily confined to closely related organisms; typical enterobacteriae have been transformed into typical members of the genus Achromobacter and more ambitious inter-family transfers to the pseudomonads are being attempted. Sancho said that Parés is preparing an extended version, in English, of his investigations and his views, for presentation at

the forthcoming National Congress of Microbiologists in Madrid. The work has been followed closely by Dr. J.L. Ingraham of the University of California at Davis, where Parés' co-worker, J. Guinea, is now working.

It may be of interest, because of the lack of English publications, to sketch the background to these recent findings as given in an incomplete set of reprints from Parés' laboratory (16-23) based upon doctoral theses by S. Hernández, R. Clotet, and J. Guinea. Reference will not be made to the papers individually as a lot of cross-checking was necessary in order to arrive at a consistent picture of what was done, and how.

Production of amino acids during bacterial growth in liquid medium: It seems that the work began with an interest in the production of amino acids during bacterial growth, already studied especially by Kinoshita in 1959, who drew attention to the possibility of amino acid synthesis on an industrial scale and to the questions that must be asked about the disturbance of biosynthetic regulatory mechanisms that must underly amino acid accumulation. Parés and co-workers first screened a number of isolates, which they carefully defined taxonomically, by growing them on a medium containing only glucose, ammonium chloride, and salts. They measured changes in dry mass, soluble protein and amino acids during growth over periods up to 264 hours: soluble protein by turbidimetry of the trichloracetic acid precipitate, amino acids by spectrophotometry of the copper complex, corrected for the ammonium chloride in the medium. During the lag phase, all cultures showed an initial appearance of amino acids and soluble protein caused by lysis (autolysis) of a substantial fraction of the inoculum. Thereafter, throughout the logarithmic phase (and beyond, according to the graphs presented), in all isolates but one, the amounts of amino acid and soluble protein per unit dry mass remained constant: amino acids were not "secreted" but were present in the medium solely as a result of the slight constant rate of autolysis. This behavior was typical of E. coli irregular; a Flavobacterium (3 strains), a member of the Achromobacter-Alcaligenes group, and Bacillus megaterium. The single exception was a strain C3 of Citrobacter intermedium (or Escherichia freundii, E. intermedium) described as being taxonomically intermediate between Escherichia and Aerobacter (Klebsiella?). This had the unique property of releasing amino acids for many hours beyond the lag period, reaching final concentrations of

about 3.2 mM/liter* or a free amino acid nitrogen to protein nitrogen ratio of about 67. The absolute rate of accumulation in the medium was dependent upon several factors; optimum values were found for the ammonium chloride concentration (aided by the presence of solid calcium carbonate) and for the size of the inoculum. Nitrogen balance calculations showed that under optimum conditions 74% of the nitrogen present as amino acids, proteins, and cell sediment is amino acid, although the data presented also show that a substantial part of the inorganic nitrogen lost from the medium was not recovered, casting some doubt on the numbers but not on the qualitative fact. The inoculum size had a very pronounced effect; within a certain range, the rate of amino acid production was independent of size of inoculum, while over a lower range it increased to a maximum value. This behavior led to the speculation, certainly on rather scanty evidence, that two modes of amino acid synthesis were involved. Some support was derived from paper chromatography which showed that at the low (inoculum-independent) rate the product was 44% alanine, increasing to 71% at the high rate. The remainder in both cases was mostly glutamic acid, with some aspartic and some basic amino acids.

Identification of amino acid secreting clones by bioautographic assay: More detailed information about the differing rates of amino acid production was obtained by streaking the cultures on agar containing the standard medium and using bioautographic procedures for detecting amino acid production by the individual colonies. A specific detector for glutamic acid is the glutamic acid requiring strain of *Leuconostoc mesenteroides* P-60 (ATCC 8042) a suspension of which in agar containing "glutamic assay broth" is poured over the colonies of *C. intermedium* and incubated. For detection of alanine, a suspension of *Pediococcus cervisiae* (ATCC 8081) can be used in the same manner using "bactoalanine assay medium," with somewhat less satisfactory results.

When a suspension of *C. intermedium* is plated and assayed for glutamic acid in this manner, about 40-50% of the colonies become surrounded by a cloudy zone of *L. mesenteroides*, showing the presence of glutamic acid

*This figure may be compared to the maximum of 224.0 reported by Samejima (cited in ref 17) for an unidentified gram-negative bacterium secreting L-alanine.

("A colonies"), while most of the remainder are clear* ("C colonies") with a few ("B colonies") that show small zones of comparatively faint cloudiness. Since the B colonies are in the minority, it is argued that the phenomenon is not simply due to statistical variation which would lead to a Poisson distribution with maximum numbers of B colonies, but a real differentiation into secreting and non-secreting cells. Generally similar results were reported with the alanine detector, the alanine positive colonies being also glutamic acid positive, as shown by distributing parts of single colonies on fresh plates. However, alanine was never absent and the distinction between A and C was never as clear cut as with glutamate.

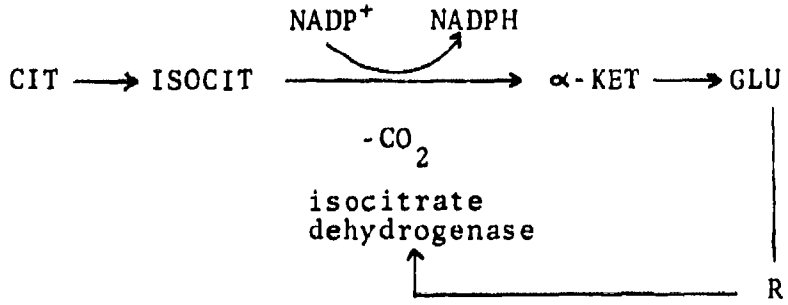
These results might suggest a preliminary scheme:

| | | | |
|---|---|---|---|
| A | B | C | colony types |
| S | ? | - | factors |
| $s^+$ | $s^\pm$ | $s^-$ | colony types |

S being used to denote an hypothetical factor responsible for amino acid secretion and $s^+$, $s^\pm$ and $s^-$ a more informative way of describing the colony types.

<u>Attempts to modify colony type by adding different substrates or intermediary metabolites</u>: The important observation was made, but not reported in detail in the papers cited here, that by addition of α-ketoglutarate to the medium all the cells could be converted into amino acid producers so that all colonies were of the $s^+$ type. On the other hand, the proportion of $s^+$ and $s^-$ colonies was unaltered by addition of glyoxalate, glutamic acid or alanine, or by replacing the glucose in the medium by a pentose, glycerol or citrate. These observations were taken to imply that the cells that give rise to $s^-$ colonies contain an active repressor of isocitrate dehydrogenase while $s^+$ colonies arise from cells in which the repressor is inactivated:

*It is stated that even the clear colonies, examined in the microscope, show the occurrence of micro-colonies of the detector organism, so that glutamic acid is never totally absent.




Some support for this scheme was found in measurements of isocitrate dehydrogenase in cells harvested from agar slants that had been inoculated with cell suspensions containing different proportions of $s^+$ and $s^-$ colony formers (obtained by the acridine orange method described below). The isocitrate dehydrogenase activity was an approximately exponential function of the percentage of $s^+$ colony formers, being about twice as great in $s^+$ cells as in $s^-$. One might perhaps have expected a greater difference in view of the large differences in glutamate production--perhaps about seven-fold--so that important questions remain unanswered. Parés assumes that the replication of the inactive represssor is controlled by an episome integrated in the bacterial chromosome, probably near the locus of a regulator gene. Thus the postulated factor S may be identified tentatively with this episome in its integrated state, S(int).

The anomalous ("paradoxal") effect of acridine orange: The episomal hypothesis of amino acid secretion would imply that (a) some cells would contain the autonomous (cytoplasmic) episome S(aut), (b) the episome would be absent from some cells, and (c) by analogy with other well established cases, S(aut) could undergo both intracellular transformation to S(int) and intercellular transfer by conjugation. Some of the evidence for the existence of S(aut) was obtained by experiments with acridine orange (AO), which is known to inhibit the synthesis of extrachromosomal DNA.

The anomalous effects of AO consist in the contrasting results of growing small inocula for short times* and

*AO 10 μg/ml, inoculum 1 - 100 cells/ml, incubation 12 hours.

large inocula for long times.* The first, if repeated, eliminates all $s^+$ colonies; the second eliminates the $s^-$ colonies; the second eliminates the $s^-$ colonies.** If either of these are subsequently grown without AO, the distribution of colony types reverts to the "normal" 40-50% $s^+$. Finally, availability of cultures with different proportions of $s^+$ and $s^-$ cells permits calculation of generation times, given as 0.9 hours and 1.25 hours respectively.

Parés uses the ideas outlined in the preceding section to explain these findings. Piecing things together, I think the argument runs somewhat as follows though rather differently expressed: Since the proportions of $s^+$ and $s^-$ colonies are amenable to modification, the two forms of the episome S must exist in some sort of "equilibrium" or steady state:

$$S(\text{auto}) \rightleftharpoons S(\text{int}) \qquad (1)$$

In addition, transfer of the episome can occur from $s^-$ cells containing S(auto) to $s^-$ cells lacking S:

$$\text{Cell 1} + (\text{Cell 2})(nS) \longrightarrow (\text{Cell 1})(n'S) + (\text{Cell 2})(n''S) \qquad (2)$$

In very dilute cultures containing AO, we are not concerned with reaction (2) because it depends upon cell-cell encounters which will occur rarely. In this situation, since AO inhibits replication of S(auto), the pool of S(auto) will be gradually attenuated, favoring "disintegration" of S(int) by reaction (1), so that the final result of continued growth under AO should be the disappearance of $s^+$ colonies and the elimination of the episome. The $s^+$ colonies do indeed disappear. Parés does not predict the elimination of the episome, nor does he discuss in this connection its reappearance when the cells are restored to a medium free of AO. He does, however, state without evidence that the number of S(auto) particles per cell is very large. It

---

*AO 10 $\mu$g/ml, inoculum $10^4$ - $10^6$ cells/ml, incubation 12-72 hours.

**Table 1 ref 18 showing the results of the first harvest from AO medium substantiates the decrease of $s^+$ at low inocula but does not show the claimed increase at high inocula unless two AO experiments that were run without controls are included.

is qualitatively conceivable therefore tha the $s^+$ colonies are completely eliminated before the episome disappears, so that under the conditions of these experiments "recovery" of $s^+$ status can occur. The experimental test of this would be to increase the number of transfers under AO beyond the 100% $s^-$ condition.

With large inocula and long incubation periods, the increase of $s^+$ colonies is taken to prove the existence of factor S in the integrated state and to show that in this condition it is not sensitive to AO. This, alone, does not explain the phenomenon, and Parés has recourse to the calculation showing that $s^+$ cells have a selective advantage because of their shorter generation time, supplemented by a supposed effect of AO in increasing the velocity (probability?) of reaction (1) in favor of S(int). Nothing is said about reaction (2) under these circumstances but it can be imagined that this occurs with increased frequency in dense suspensions prior to the attenuation of S(auto) thus increasing the number of cells containing S(auto) and thus potentially able to be transformed to the $s^+$ state. Careful calculation of the quantitative consequences of these mechanisms are evidently needed, and possibly a critical re-examination of the experimental basis.

Episomal conjugation sensitive to mechanical agitation: So far the phenomena described do not depend qualitatively upon reaction (2) although this, if it occurs, must influence the distribution of S and therefore the colony types. Direct proof of intercellular transfer by conjugation lies in the sensitivity of the colony type distribution to mechanical agitation, referred to several times but not described in detail in the papers available to me.

Interspecific transfer of episome: In principle reaction (2), intercellular transfer of free episome, could occur between species. This would be an efficient means of incorporating new material in the genome of another cell because of the supposed rapid intracellular replication of S(auto) and the high probability of integration, estimated at $3 \times 10^{-2}$ per cell x generation.

Success was claimed in transferring the amino acid secretion factor S(auto) from C. intermedium C3 to Paracolobactrum intermedium (ATCC 1166), which had been tested for amino acid production with consistently negative results. Mixtures of the two organisms with a ten-fold predominance of P. intermedium were incubated

for two hours at 30°C, diluted, and streaked on McConkey plates. Since P. intermedium ferments lactose only very slowly, the colonies were white while those of C. intermedium were pink and thus easily distinguishable. When the P. intermedium colonies were resuspended, streaked on agar, incubated for 25-30 hours, and overlaid with Leuconostoc, colonies positive for glutamic acid were found in five experiments out of six, involving 47 $s^+$ colonies out of a total of 464. Thus interspecific transfer seems to have been demonstrated. Some of the secreting colonies were transferred, grown for 24 hours, and again plated for amino acid assay. Of 200 colonies so examined all were non-secretors, $s^-$. This rapid elimination of the S(int) episome casts some doubt upon the interpretation, if not the accuracy, of the original observations that seemed to show interspecific transfer of the ability to secrete glutamic acid.

Conclusion: This brings us back to the starting point, where we reported the rather far-reaching views expressed on the possibilities of information transfer among comparatively remotely related microorganisms. The brief survey just completed, without reference to the work of other laboratories, has left me with the feeling that there are many loopholes which make the episomal interpretation of the data somewhat less than completely satisfactory. In the hope that this may be due to the incompleteness with which the data have been abstracted from the unavailable doctoral theses, the news that Parés is preparing an authoritative summary is very welcome.

## IV. THE UNIVERSIDAD AUTÓNOMA DE BARCELONA DEPARTMENT OF MICROBIOLOGY

The Universidad Autónoma is one of three such new universities in Spain, the others being in Madrid and Bilbao. Recent decisions by the Ministry of Education are intended to place all the Spanish universities, the new and the old, on an equal footing, with some liberty and autonomy in a narrow sense. The new university in Barcelona is in its fourth year. It is housed in a converted hospital building, an uninspired but spacious structure in the grounds of the Santa Cruz Hospital at San Pablo. In a pleasant new temporary building nearby the Department of Microbiology, now three years old, occupies the first (upstairs) floor. It is headed by Professor A. Foz with two associate professors and six research associates with doctorates in pharmacology, biochemistry and microbiology. Professor Foz was kind enough to describe his research interests which center around the subject of brucellosis.

Brucellosis is prevalent in Spain, with at least 6000 human cases. Professor Foz, with 20 years experience in this field, is a WHO expert in the diagnosis and serology of Brucella, including antigen standardization and interpretation of the Coombs test for B. abortus. His U.S. connections include Dr. Wesley Spink of the University of Minnesota Medical School, who has visited the Department.

Problems of diagnosis arise in patients who have received vaccination against cholera (over a million in Spain), because their sera contain antibrucella agglutinims. Professor Foz is investigating the nature of these antibodies in relation to those occurring in brucellosis. Another microorganism, Yersinia enterocolitica, related to Pasteurella pestis, also causes formation of antibodies active against Brucella. The triangular set of Vibrio-Brucella-Yersinia relationships is being studied, including some work on the non-pathogenic Vibrio Nag which is found to be antigenically quite different from V. cholerae. Other organisms causing urinary and enteric infections are being studied; the salmonelloses are very common, and shigellosis rather uncommon, in Spain. All this is being done in close collaboration with the Municipal Hospital for Infectious Diseases.

Antibiotic testing: A new antibiotic, tobramicina, is being tested for the Lillie Company. Foz finds all strains of Pseudomonas to be very sensitive in vitro. The first in vivo tests are being made in the U.S.

This appears to be an active young department whose accomplishments have perhaps received less than their proper recognition in the outside world because of the language difficulty; Professor Foz, a friendly and courteous man, speaks French well but is uncomfortable with English.

## V. SUMMARY AND COMMENT

A few of the microbiological research projects engaging the attention of scientists in Spain are reviewed, in varying depth, on the basis of visits to five laboratories and perusal of some of their publications. Topics discussed include: (1) The immunosuppressive effects of metabolites of Pseudomonas (Instituto "Jaime Ferrán", Madrid). (2) Search for viruses which interfere with infection by African swine fever viruses (same). (3) Pathogenic non-reverting L-forms of pathogenic bacteria (same).

(4) Localization of antibiotic action within the protein synthesizing system (Instituto de Biologia Celular, Madrid). (5) Control of leptospirosis (University of Barcelona, Faculty of Medicine). (6) Interspecific transfer of episomes for amino acid "secretion" (University of Barcelona, Faculty of Science). (7) The Brucella-Vibrio-Yersinia cross-reacting system ("Autonomous" University of Barcelona).

The projects appear to be carried out with much competence although it is likely that they would benefit by closer and more frequent contact with ongoing research in some of the major centers, none of which can be said to be situated in Spain. In addition to a certain isolation, the microbiologists work under crowded conditions with inadequate support for modern equipment and technical help. On the other hand, social mobility is less marked than elsewhere and a well-trained assistant is likely to remain available as long as the position can be maintained financially.

The microbiologists visited were informed about the functions of the European Research Office and several expressed interest in submitting informal research contract projects for further discussion. Negotiation of research contracts on some of the topics listed above could be beneficial to the U.S. Army research program while assisting the Spanish scientific community in a modest way, both materially and in promoting increased contact with scientific establishments in the United States and elsewhere. Comments and expressions of interest from the recipients of this report are cordially invited.

## VI. REFERENCES


1. Patronato Santiago Ramón y Cajal: Centro de Investigaciones Biologicas. Memoria 1969. (Madrid).

2. ib., Memoria 1970.

3. Hottle, G.A. 1971. Microbiology in Spain. Office of Naval Research, London, Report R-11-71, 30 March.

4. Bateman, J.B. 1972. Surveys of Thalassemia and Other Blood Defects in Spain, European Research Office Technical Report 7.

5. Portolés, A., and Perez-Ureña, M.T., 1963. Control of infections by Pseudomonas. Proc. III Internat. Congr. Chemother., C-47, 519-522. Georg Thieme Verlag, Stuttgart.

6. Portolés, A., Espinosa, M., and Hidalgo, A. 1971 Penicillin and polymyxin effects on the chromogenesis of Pseudomonas aeruginosa strains. J. Antibiotics, 24, 266-269.

7. Pérez Ureña, M.T., Espinos, M., López, R., and Portolés, A. 1972. Immunosuppressive effects produced by some extracellular metabolites from Ps. aeruginosa strains. Zbl. Bakt., in press.

8. Rubio, N., and Lopez, R. 1971. Purification of Pseudomonas aeruginosa endotoxin by gel filtration on Sepharose 4B. J. Chromatog., 57, 148-150.

9. Gil-Fernández, C., Alonso, L., García-Gancedo, A., and Ronda, E. 1971. Hemadsorcion inspecifica y especifica sobre celulas de riñon de cerdo normales e infectadas con el virus de la peste porcina africana. Microbiol. Españ., 24, 23-38.

10. Gancedo, A.G., Alonso, N.L., Gil-Fernández, G., Ronda, E., Vilas, P., and Ronda, C. Undated proof. Interferencia inducida por el virus de la peste porcina africana (PPA). "Memo 112," pages 1-6.

11. Beltrá, R. and Rodriguez de Lecea, J. 1971. Aseptic induction of the crown-gall tumors by the nucleic acid fraction from Agrobacterium tumefaciens. Phytopath. Zeitschr., 70, 351-358.

12. Rubio-Huertos, M., Vela, A., and López-Abella, D. 1967. Crystalline arrays of spherical particles in turnip yellow mosaic virus-infected cells. Virol., 32, 438-444.

13. Rubio-Huertos, M. 1968. Further studies on ultrastructure of plants infected with Petunia ringspot virus. Protoplasma, 65, 465-476.

14. Vazquez, D., Staehelin, T., Celma, M.L., Battaner, E., Fernández-Muñoz, R., and Monro, R.E. 1970. Peptide bond formation in protein biosynthesis and the action of antibiotics. FEBS Symp., 21, 109-130.

15. Vazquez, D. 1969. Mechanism of action of antibiotics. Ann. Rep. medicinal Chem., Chap. 15, 156-169. (Academic Press, New York).

16. Clotet i Ballús, R., and Parés i Farràs, R. 1967. Dos tipus de segregació d'aminoàcids per un coliform (Escherichia intermedia (3). Treballs Soc. Catalana Biol., 23, 61-69.

17. Clotet, R., Guinea, J., and Parés-Farràs, R. 1968. Segregacion de aminoacidos por una cepa de C. intermedium. Microbiol. españ., 21, 155-173.

18. Guerrero, R., and Parés i Farràs, R. 1969. Effecte paradoxal del taronja d'acridina en relació a l'acció d'un factor episòmic de Citrobacter intermedium C3. Treballs Soc. Catalana Biol., 27, 83-87.

19. Urgell, J.B., and Parés i Farràs, R. 1969. Activitat isocitricdeshidrogenàsica en preparats solubles de differents poblacions de Citrobacter intermedium C3. ibid., 91-97.

20. Clotet, R., Guinea, J., and Parés-Farrás, R. 1969. Producción de alanina por fermentación con Citrobacter intermedium C3. Revista españ. Fisiol., 25, 41-54.

21. Parés-Farrás, R., and Guinea, J. 1969. Transferencia interspecifica del factor episomico que condiciona la segregacion de glutamato en Citrobacter intermedium C3. Anales Estacion exp. Aula Dei, 9, 287-296.

22. Guinea, J. 1970. Analisis colonial de la produccion de acido glutamico por Citrobacter intermedium C3. Microbiol. españ., 23, 13-22.

23. Hernández, S. 1970. Heterogeneidad colonial de Citrobacter intermedium C3, en relacion con la segregracion de alanina. ibib., 23, 139-147.

## VII. DISTRIBUTION

| | |
|---|---|
| 1 | ERO, CO |
| 2 | AFEB (Prof G.J.Dammin; Col B.W. Prior) |
| 1 | AMEMB London (Dr. A.G. Mencher) |
| 1 | AMEMB Paris (Dr. E.L. Piret) |
| 1 | AMEMB Rome (Dr. O.H. Ganley) |
| 9 | AMRDC (Col R.F. Barquist (2)); Brig Gen R.R.Taylor (2); Maj R.M. Glickman; LTC D.W. Sample; LTC J.L. Hatfield; LTC G.B. Randolph, Jr; Col J.B. Young) |
| 1 | BAMC (LTC B.A. Pruitt, Jr) |
| 2 | BSRL (Dr. J.E. Uhlaner; Dr. J. Zeidner) |
| 1 | CRL |
| 6 | DARD (Gen C.D. Daniels, Jr, ARZ-A (2); ZA; Dr. C. Lamanna ARL-M; Col G. Rapmund, ARL; Dr. E.M. Sporn, ARL-P |
| 3 | EOAR (LTC R.F. Prueher; LTC R.B. Wallace; Maj W.H. Inge, Jr) |
| 2 | FASEB (Dr. C.J. Carr; Dr. E.L. Hess) |
| 1 | FDA (Dr. R.A. Littleford) |
| 1 | MEDEDCG (Brig Gen R.M. Hardaway III) |
| 2 | MRL (Director; Dr. C.C. Hassett) |
| 1 | NAMRU-3 (CO) |
| 1 | NASA |
| 2 | NATO (Dr. E.G. Kovach) |
| 2 | NBL (Dr. R. Valeri) |
| 2 | NBRL (Dr. G.A. Hottle) |
| 2 | NEPMU-7 (Cmdr W.G. Miner) |
| 1 | NIH (Dr. M.D. Leavitt, Jr) |
| 3 | NSF (Dr. E.R. Sohns (2); Dr. B. Bartocha) |
| 5 | ONRL (Dr. E.I. Salkovitz; Dr. R.R. Sonnenschein; Dr. J. Foss; Capt A.J. van Tuyl, Jr; Dr.A.W.Frisch) |
| 2 | OTSG (LTG H.B. Jennings, Jr) |
| 1 | RDRD |
| 1 | RDRF (Col B.C.A. Walton) |
| 1 | RDRL (Mr. H.F. Weiler) |
| 2 | RIEM (Col L.G. Jones) |
| 2 | USAARL (Col R.W. Bailey; Dr. E.J. Baldes) |
| 1 | USAMLO (LTC M.C. Duffy) |
| 1 | USAMRNL |
| 5 | USANL (Dr. S.D. Bailey; Dr. H.L. Jacobs; Dr. G.R. Mandels; Dr. J.F. Oesterling; Dr. D.H. Sieling) |
| 5 | USARIID (Col W.R. Beisel (3); Dr. A. Buzzell; Col D. Crozier) |
| 2 | USPHS (Dr. R. Freckleton; Dr. B. Blood) |
| 1 | WHO (Dr. W.P. Faulk) |

| | | |
|---|---|---|
| 19 | WRAMC-WRAIR | (LTC C.R. Angel; Dr. M. Artenstein; Dr. J.F. Barbaro; Col M.E. Conrad; LTC R.T. Cutting; Dr. B.L. Elisberg; Dr. E.H. Fife, Jr; Dr. A.D. Glinos; Dr. F.E. Hahn; LTC H.C. Holloway; Col T.H. Lamson; Dr. H.E. Noyes; LTC R.A. Olsson; Col W.E. Rothe; Col P.K. Russell; Dr. E.H. Sadun; LTC P.K. Hildebrandt; Dr. T.R. Sweeney; Dr. S. Vivona) |
| 1 | AMRDC | (Col K.R. Dirks) |

## ANNEX

### The Seven Research Institutes of the Centro de Investigaciones Biologicas, Madrid

(abridged and translated from ref 2)

#### Institut "Santiago Ramón y Cajal"

##### Department of Biophysics

Cytology and Comparative Histology
Electron Microscopy
Comparative Neuroanatomy
Neuroangiology
Pathological Anatomy of the Nervous System
Histochemical Laboratory

#### Instituto "Jaime Ferrán"

##### Department of Virology

Plant Viruses
Animal Viruses

##### Department of Bacteriology

Biology of Bacterial Infections
Symbiotic Nitrogen Fixation
Direct Nitrogen Fixation
Bacterial Physiology and Genetics

##### Department of General Microbiology

Yeasts
Microbiological Technique
Phytopathological Microbiology
Microbial Chemistry
Applied Mycology

##### Department of Protozoology

Protozoal Cytology
Ecology

Instituto "Arnaldo de Vilanova"

History of Spanish Medicine

Instituto "Gregorio Marañón"

Department of Metabolism

Pancreatic Hormones
Immunochemistry of Hormones
Molecular Biology
Molecular Genetics

Department of Experimental Endocrinology

Thyroid
Steroids
Biology of Photosynthetic Organisms

Instituto de Biología Celular

Department of Biochemistry

Biosynthesis of Proteins
Biochemistry of Membranes

Department of Cytology

Cell Structure
Cell Physiology

Department of Cell Chemistry

Microbial Physiology
Enzymatic Regulation

Instituto de Enzimología

Department of Enzymatic Regulation

Biocatalysis
Metabolic Regulation

Department of Microbial Enzymology

Biochemical Genetics
Biosynthesis of Enzymes

## Instituto de Genética y Antropología

### Department of Anthropology

Human Somatology
Human Genetics

### Department of Dynamic Genetics

Population Genetics
Developmental Genetics

### Department of Cytogenetics

Plant Cytogenetics
Animal Cytogenetics