Establishment of the Indigenous Microbial Flora of the Intestines in the Course of the Evolution of Vertebrates

It is highly probable that many parasites have evolved contemporaneously to their hosts, parasitism being a very long-standing phenomenon and an early biological trait of vertebrates (CAMERON[1]). While investigations of comparative parasitology have been carried out, e.g. on sclerostomata, no serious attempt has been made to evaluate the association of the intestinal microbial flora with the evolutionary development of vertebrates: it is well known that the indigenous flora inhabits the gut of mammals and also of birds; but information appears scanty or misleading in the case of cold-blooded animals.

From the alimentary tract of the common gartersnake (*Tamnophis sirtalis sirtalis*), MERGENHAGEN[2] isolated bacteria belonging to eight genera and stated that 'there seems to be little if any difference in the bacterial flora of the alimentary canal of poikilothermic and warm-blooded animals'. His conclusion, being gratuitously extended to poikilothermic animals in general, seems wrong, since a true microbial flora appears to be absent in the intestine of fish, which, after brief periods of fasting, tends to become sterile. 'There are no common bacterial commensals in the intestine of fish, but the presence of a bacterial flora depends solely upon the recent intake of food and water' (MARGOLIS[3]).

Similar conclusions are shared by POTTER and BAKER[4], GUÉLIN[5] and HUGGINS and RAST[6], only LISTON[7] suggesting a hypothetical analogy between the coliform population of mammalian gut and some intestinal groups of genus *Vibrio*, isolated from specimens of *Pleuronectes microcephalus* and *Raja* sp. But this suggestion awaits confirmation.

Our investigations were directed towards the study of the missing ring of the chain, i.e. amphibia, in particular those living chiefly in an aquatic environment. Comparative experiments were performed on newts (*Triturus cristatus carnifex*), frogs (*Rana esculenta*) and, for reference, a teleost, the goldfish (*Carassius auratus*). Twelve adult specimens of each species were acclimatized in running fresh-water at 18°C. One-third of the animals were killed 2–4 h after their last feed; one-third after being kept fasting for 7 days; the remaining third after 2 weeks of starvation. The alimentary canal was isolated aseptically and slit in the anterior, middle and posterior one-third regions. From the intestinal contents several slides were prepared and evaluated.

In the actively feeding fish several bacteria were observed, distributed throughout the intestines; while in the fasting fish the gut appeared completely germ-free or showed rare, isolated forms, chiefly in the anterior one-third region.

In newts a rich and multiform microbial flora was observed, much more abundant in the hind-gut; there seemed to be little if any difference in the quantity and quality of bacteria between actively feeding animals and fasting ones.

In the frog, either recently fed or starved, the intestinal flora was constantly present and very copious. In the posterior tract of the gut it closely resembled the colonic flora of mammals.

Two other groups of goldfish and frog (5 specimens for each group) were fasted for 2 weeks. Then the stools of the last 24 h were collected and homogenized and the bacteria counted by numbering the colonies grown in agar plates inoculated with serial dilutions. The average number of bacteria found in the 24h faeces of each goldfish was $2 \cdot 10^8$; the corresponding number for each frog was $3 \cdot 10^{20}$: that is, the microbial population present in the 24h faeces of a fasting frog is of the order of $1.5 \cdot 10^{12}$ times more than in the goldfish.

It seems, therefore, that terrestrial vertebrates, from amphibia to man, have evolved in intimate association with a complex indigenous flora of the intestine. On the contrary, in the intestine of fish, a true microbial flora appears to be absent or restricted to a scanty and labile form.

The explanation of such differences will be examined in a later paper.

Riassunto. È stato osservato che la flora microbica indigena intestinale, praticamente assente nei Pesci, si trova associata stabilmente nei Tetrapodi, a partire dagli Anfibi Urodeli e Anuri.

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Three Antifungal Polypeptides from *Bacillus subtilis*

Among the antifungal polypeptides synthesized by different strains of *Bacillus subtilis*, the bacillomycins have been grouped together due to their great similarity in many respects: molecular weight, amino acid composition, pK, solubility, biological action, etc.[1].

In this communication we describe three antifungal polypeptides obtained with a strain of *B. subtilis* derived from the original producer of bacillomycin A[2]. These compounds have several properties in common with the members of the bacillomycin family.

B. subtilis DINR 49-4 was grown in Povitsky flasks on potato broth admixed with 2% of glucose and 0.4% of asparagine. After 15 days' incubation at 25°C the culture was sterilized, brought to pH 3 with concentrated hydrochloric acid and left overnight at 4°C. The turbid liquid was stirred with Hyflo-Supercel (Johns Manville) and centrifuged. The solids obtained were extracted with hot

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ethanol, the extract was dried at 40–50°C and this residue was suspended in hot methanol. The methanolic solution was dried and the residue was reextracted in the same way twice more. The final solution was precipitated by addition of ether and this precipitate was dissolved in methanol and reprecipitated with ether at least 4 more times. The yield was usually 300 to 500 mg of a brown powder per litre of fermented broth. This material was further purified by counter current distribution in the system: sodium citrate buffer 0.15 *M*, pH 6.2:methanol: benzene:*n*-butanol (6:8:8:3; v/v). At 1392 transfers it was possible to obtain fraction I (K = 0.12), which showed a minimum inhibitory concentration (mic) of 4 μg/ml on *Neurospora crassa* DINR 136. With the material remaining in the counter current distribution apparatus, two further cuts could be obtained at 2871 transfers: fraction II (K = 0.07; mic: 5 μg/ml) and fraction III (K = 0.06; mic: 12.5 μg/ml).

The amino acid composition of the three fractions was remarkably similar: (Asp_3, Glu_1, Pro_1, Ser_1, Tyr_1). This analysis was performed on total acid hydrolysates by the MOORE, SPACKMAN, and STEIN method[3]; the weight recovery was 80 to 92%. The minimum molecular weight of the three fractions was calculated, on the basis of one tyrosine residue per mole, by light absorbancy measurements at 275 mμ and also by determinations of amide nitrogen (assuming one amide group per mole). The values obtained were coincident with the one derived from amino acid analysis. In no case could a free amino group be detected by reaction with 1-fluoro-2,4-dinitrobenzene; the only derivative obtained after acid hydrolysis was O-DNP-tyrosine.

In spite of the chemical similarities indicated above, the three fractions showed significant differences in their biological activities (Table). These can only be ascribed to structural reasons not clear at present. Although the amino acid analysis, the N-terminal studies and the consistent results obtained for the molecular weight indicate a reasonable purity of these preparations, it must be stressed that their distributions were not strictly coincident with the theoretical ones for pure substances. Experiments are now in progress to investigate further the chemical structure of these compounds.

According to the present results these antibiotics are very similar to those of the bacillomycin group. One significant difference, though, is the presence of proline in their molecules[4].

Comparative antimicrobial spectra of the three polypeptides isolated

Organism	Minimum inhibitory concentration (μg/ml) Polypeptides		
	I	II	III
Corynebacterium diphtheriae G-12-6	32	>32	>32
Neurospora crassa DINR 136	4	5	12.5
Neurospora crassa 73a	6	6	25
Penicillium chrysogenum	>32	4	>32
Trichophyton tonsurans	16	32	8
Trichophyton rubrum	>32	32	>32
Alternaria solani	40	>40	40
Rhizoctonia solani	>40	40	>40
Mucor racemosus	>40	40	>40
Botrytis cinerea	>40	40	>32

Zusammenfassung. Drei antimycotische Polypeptide von gleichem Mindestmolekulargewicht mit identischer Aminosäurezusammensetzung (Asp_3, Glu_1, Pro_1, Ser_1, Tyr_1) aus *B. subtilis*-Kulturen wurden isoliert. In keinem von ihnen wurden Aminogruppen gefunden, die sich mit dem Reagenz von Sanger verbinden. Trotzdem wurden Unterschiede im biologischen Verhalten gefunden. Alle Resultate (Prolin ausgenommen) deuten auf eine Verwandtschaft mit der Gruppe der Bacillomycine.

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Sperm Nucleus of *Clibanarius longitarsis*

The sperm nucleus of *Clibanarius longitarsis*, a hermit crab, reacts negatively to all the histochemical tests applied for the demonstration of DNA – an essential constituent of every nucleus. However, the nuclei of spermatogonia, spermatocytes (Figure 1), nutritive cells and interstitial cells show the presence of DNA in them.

The nuclei of early spermatids show only a feeble Feulgen reaction[1] for DNA, but as the process of spermateleosis proceeds, even this small amount of detectable DNA gradually becomes undemonstrable, so that the mature spermatid nuclei are virtually Feulgen negative (Figure 2) and the sperm nuclei totally lack demonstrable DNA. Indeed there are no traces whatsoever of demonstrable DNA in any region of the ripe sperm.

The spermatid nucleus appears structureless with only 2–3 lightly stained granules in iron haematoxylin[2]. The sperm nucleus appears pale and structureless in Lewitsky-saline fixed, haematoxylin stained tissue, but Zenker fixed material reveals a large number of lightly stained granules. These granules stain with crystal violet (Figure 3) and they are particularly well revealed in acid haematein test after pyridine extraction[3] (Figure 4), due to the presence of proteins in them.

There is no visible change in the spermatid nucleus for a long time. After the differentiation of other sperm struc-

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