The sequence of bile pigment formation heme → biliverdin → bilirubin occurs in human beings, most of the mammals and also in reptiles and fishes[9,10]. However, in some vertebrates like chickens and possibly other birds, the sequence seems to stop at the biliverdin level due to lack of biliverdin reductase[11] both in liver and spleen, and consequently those species excrete mostly unconjugated biliverdin in bile[10,12]. A similar explanation cannot be suggested for the origin of biliverdin in buffaloes' milk, since biliverdin reductase activity is present in spleen, though this enzyme has been reported to be absent from the liver of ruminants[8]. To explain the conversion of ^{14}C bilirubin of blood serum to ^{14}C biliverdin, which was recovered in milk[6], the oxidation of bilirubin in buffalo mammary tissues appears to be a possible mechanism. Since biliverdin does not occur in normal sera[13], the presence of bilirubin in buffaloes' milk is attributed to the oxidation of bilirubin in the mammary tissues as shown in the present study. In the light of the above mentioned facts, it may be surmised that the biliverdin reductase like activity from buffalo mammary tissue is different from that observed in liver, spleen and placenta. To explain the occurrence of biliverdin in buffalo milk we suggest that the oxidation of bilirubin in buffalo mammary tissue is catalyzed by an oxidase. It is of interest to note that recently bilirubin oxidase has been characterized for the first time in the microorganism *Myrothecium verrucaria*[14-16].

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Changes of the cell components of *Escherichia coli* and *Pseudomonas fluorescens* in deficient medium

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Summary. Strains of *Escherichia coli* and/or *Pseudomonas fluorescens* prelabeled with 14-C-glutamic acid show differences in the marker distribution in the cell components. Moreover, there are essential distinctions in the trend and rate of degradation of macromolecular fractions under starvation conditions.

The physiological changes occurring in bacterial cells exposed to environments without essential exogenous substrates are usually studied with strains of *Escherichia coli*[1-3]. We used, for comparison, *Pseudomonas fluorescens* and examined the cell component changes during exposure of bacteria to a medium without a carbon source. The main reason for performing this work is that the phenomenon of microbial survival in nutrient-limited natural waters occurs frequently[4,5], but the processes are understood very poorly. The results presented here on the distribution of radioactive label originating from 14-C-precursors into different macromolecular fractions of bacteria before and after starvation supplement our previous observations[6,7].

Materials and methods. Escherichia coli strain CCM 2260 and *Pseudomonas fluorescens* strain CCM 2115 from the Czechoslovak Collection of Microorganisms of the J.E. Purkyně University Brno were used for experiments. The overnight inoculum (12 h) was prepared with bacteria taken from agar slants.

For preparation of labeled culture, 14-C-glutamic acid or 14-C-valine (4 kBq per 1 ml of media) was added to 3000 ml of Proteose peptone medium (Oxoid). During

Table 1. The 14-C label remaining in 14-C-prelabeled cells of *E.coli* and/or *P.fluorescens* after starvation in Davis mineral medium without carbon source

Strain	Without TCA precipitation (cpm)			TCA precipitate (cpm)		
	0 h	72 h	168 h	0 h	72 h	168 h
E.coli	32880 (100)*	22070 (67.1)	16520 (50.2)	30460 (92.6)	18930 (57.6)	10570 (32.1)
P.fluorescens	1560 (100)	1120 (71.8)	764 (49.1)	1510 (100.6)	840 (53.8)	600 (38.5)

*In brackets the counts are expressed as percentage of label which remains in cells or TCA precipitates when compared to the total cellular label before starvation.

exponential growth of aerated cultures[8] and at an OD of 0.3 (OD_{575}) cells were centrifuged and transferred into Davis mineral medium (DMM) without glucose and citrate[9]. The suspension of cells was incubated at 30 °C without aeration. 1 l aliquots were withdrawn at 0, 72 and 168 h, respectively, for chemical fractionation as described by Sutherland and Wilkinson[10]. In a sequential extraction process polysaccharides, lipids, proteins and nucleic acids with polyphosphates were separated using cold trichloroacetic acid (TCA), methanol, chloroform and hot TCA, respectively. The cpm from samples of cells or cell fractions were measured using a liquid scintillation method[7] on a Tricarb apparatus. In measurements of the radioactivity the method described by Albright et al.[11] was used. For determination of the remaining total label, aliquots of the cell suspension were applied to filter disks (Whatman 3MM) and dried without TCA precipitation. The colony forming units (CFU) were estimated by 48 h incubation at 30 °C of diluted culture samples on Proteose peptone agar (Oxoid) medium[12]. 99% confidence limits for mean values were computed as described by Clarke[13].

Results. Cultivation of *E. coli* cells in medium with 14-C-glutamic acid under the experimental conditions (Oxoid proteose peptone) resulted in the incorporation of most of the 14-C into proteins (50%) and lipids (more than 35%), whereas in *P. fluorescens* the main share of the marker was found in lipids (72%) and polysaccharides (fig.).

In both bacterial strains 9% of the marker was incorporated in nucleic acids. This value remained constant throughout the starvation experiment, the only exception being the 168 h sample with *P. fluorescens*, where the cpm decreased to half of the original level. In the time interval 0–72 h, in the absence of exogenous carbon, both organisms degraded their polysaccharides and, in the case of *P. fluorescens*, their lipids as well.

In the course of starvation some macromolecular resynthesis was observed. In the case of *E. coli* there was an approximately 15% increase of marker level in the lipid fraction, and for *P. fluorescens* a temporary increase of cpm in the protein fraction was observed. When labeling with 14-C-valine the incorporation of 14-C also differs with the 2 bacteria. In *E. coli* there was 20-times more marker than in *P. fluorescens* (table 1). The total degradation rate is approximately the same for each organism, irrespective of the cpm estimation, with or without TCA precipitation.

The results in table 2 illustrate the survival of the 2 bacteria during exposure to an environment without exogenous source of carbon and without aeration.

In the cultivation experiments mineral media were used with glucose, glutamic acid and valine, respectively as the sole sources of carbon. The results show that strains of *E. coli* CCM 2260 and *P. fluorescens* CCM 2115 are not able to utilize valine as the sole source of carbon and energy. For glutamic acid, doubling times were 0.8 h *(E. coli)* and 1.3 h *(P. fluorescens)*. On the other hand, glucose alone allowed growth of *E. coli* and *P. fluorescens* with doubling times of 0.7 and 1.7 h, respectively.

Discussion. Fundamental differences between *E. coli* and *P. fluorescens* are shown in the distribution of the 14-C label (from 14-C-glutamic acid) incorporated into different cell components (fig.). The incorporation into the protein fraction by *P. fluorescens* was very low, whereas *E. coli* incorporated a high level of the labeled precursor into this fraction. This is in accordance with the doubling times measured in the cultivation experiments. Similarly, using another precursor (14-C-valine), the total 14-C incorporation is also higher in *E. coli* than in *P. fluorescens* (table 1).

Distinct differences between the 2 species were also found in their degradation rates for various components labeled with 14-C-glutamic acid. Exposure of *E. coli* for 72 h in carbon-free medium caused only polysaccharide degradation (in accordance with Dawes'[1] results) but in the case of *P. fluorescens*, lipids were degraded as well.

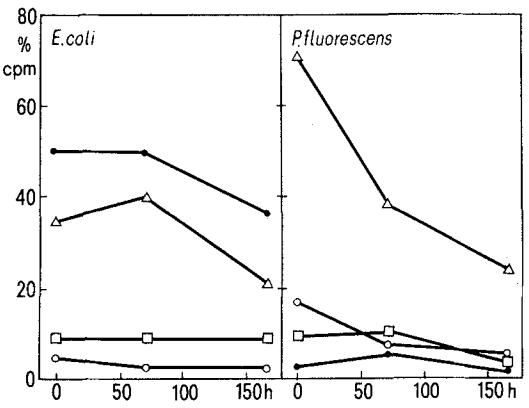
The results with 14-C-valine prelabeled cells demonstrated relatively fast degradation of proteins in both bacterial strains. This corresponds to our previous observation, obtained with *E. coli*[8], but contradicts the data of the figure. The striking difference in glutamic acid and valine utilization in metabolic processes must be taken into consideration as well as redistribution of label due to turnover of macromolecules. Also, differences in the utilization of the given precursors in cell energy production have to be considered; glutamic acid can be used as an energy source and can therefore contribute to the stimulation of stressed cells[6]. It is worth noting the similar degradation rate of proteins by both bacteria prelabeled with 14-C-valine. This suggests a similarity in the proteolytic processes of *P. fluorescens* and *E. coli* in the absence of exogenous energy sources and where only limited endogenous sources are present. The presence of glutamic acid in the pool or in incorporated forms reduces this deficiency and causes a change in turnover rates in both *E. coli* and *P. fluorescens*.

The transient increase in protein levels in *P. fluorescens* and in lipids by *E. coli* demonstrates the potential for substantial macromolecular synthesis in bacterial cells exposed to limiting nutrient conditions. Similar effects were reported in our previous papers[14,15], where, in the initial phase of the

Table 2. Survival of *E. coli* and/or *P. fluorescens* during 168 h of exposure without aeration at 30 °C in Davis mineral medium without carbon source

Strain	CFU* in 10^6/ml		
	0 h	72 h	168 h
E. coli	190 ± 85**	136 ± 59	96 ± 58
P. fluorescens	198 ± 69	151 ± 51	96 ± 37

*CFU, colony forming units obtained after 48 h cultivation of culture samples on Proteose peptone agar (Oxoid) medium at 30 °C.
**The confidence intervals of means at 99% significance level were computed from 6 measurements by the method of Clarke[13].



Changes of the distribution of 14-C (in cells prelabeled with 14-C-glutamic acid) into different cell components during starvation of *E. coli* and/or *P. fluorescens* in Davis mineral medium without carbon source. Polysaccharides, lipids, proteins and nucleic acids with polyphosphates were separated in a sequential extraction procedure using cold TCA, methanol, chloroform and hot TCA, respectively. The level of macromolecular fractions in cells is expressed by means of cpm, as a percentage of the total value: ○—○, polysaccharides; △—△, lipids; ●—●, proteins; □—□, nucleic acids with polyphosphates.

starvation process, the number of colony forming units increased. Taking into account the similar trend in the survival of both bacteria when lacking aeration in DMM at 30 °C, there is nevertheless a substantial difference in the changes of degradation rates and concentrations of the cell components. This is caused by differences in the characteristics of the 2 species, primarily in the regulation of metabolism and in the utilization of different metabolic pathways under starvation conditions.

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The presence and partial characterization of carbohydrase enzymes in the gut of *Callosobruchus maculatus*

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Summary. Carbohydrase activity has been demonstrated in homogenates of the alimentary tract of *C. maculatus:* β-D galactosidase > α-D glucosidase > α-D galactosidase > β-D glucosidase activity. The effects of pH, temperature and substrate concentration on β-D galactosidase, α-D glucosidase and α-D galactosidase activities are described.

Callosobruchus maculatus F. is a major storage pest of the cowpea, *Vigna unguiculata*[1]. To date studies on this insect have tended to concentrate on ecological and behavioral aspects[2-7] whilst the physiology and biochemistry of *C. maculatus* and indeed the bruchids as a whole, has been virtually ignored.

Whilst digestive glycosidases have been reported in many insect species[8], very little information of a quantitative nature is available concerning their biochemical properties[9]. Hydrolysis of various carbohydrates by insects can be explained by supposing that carbohydrate specificity is dependent on the nature of the substrate, in particular its glycosidic bond and the α or β form of the linkage[10]. This hypothesis postulates the existence of 5 basic enzymes capable of hydrolyzing all oligosaccharides and glycosides based on glucose, galactose and fructose. However, one or more of these glycosides may be absent from the digestive fluid of a given species[11]. Fraenkel[12] used Weidenhagen's[10] hypothesis to explain digestion of all di, tri-saccharides and glucosides, utilized by adult *Calliphora vicina,* by the presence of only 2 gut enzymes (α-glucosidase and α-galactosidase). Whilst this hypothesis has been criticized[11,13-15], Bongers[16] and Wenzyl[17] have more recently used the idea of an unspecific α-D-glucosidase to explain their results from *Oncopeltus fasciatus* and *Calliphora erythrocephala.* On this basis it was considered valid to assay the activities of the 4 main classes of oligosaccharidases (α- and β-D-glucosidase, and α- and β-D-galactosidase) using p-nitrophenyl glycosides as 'generalized' substrates.

The present paper describes the presence and partial characterization of oligosaccharidase enzymes from the alimentary tract of *C. maculatus* and is intended as a basis for future physiological and biochemical studies.

Materials and methods. A culture of *Callosobruchus maculatus* originating from Campinas, Brazil, was reared and maintained on seeds of *Vigna unguiculata* at 28 ± 0.5 °C and 60% relative humidity. The photoperiod was arranged to provide 12 h light and 12 h dark.

Sexually mature animals of both sexes were killed by decapitation and their alimentary tracts removed by dissection under ice-cold (ca. 4 °C) distilled water. Homogenization was carried out in distilled water in a Potter-Elvehjem homogenizer with a Teflon pestle (clearance 0.1–0.15 mm) with 20 passes of the plunger at 1000 rev/min; the homogenization tube was surrounded by ice throughout this procedure. The resulting homogenate was then centrifuged at 9000 × g for 5 min at 4 °C in a Haematocrit centrifuge and the supernatant retained for enzyme assay. In general the alimentary tracts from 110 animals were homogenized in 1.5 ml distilled water. All homogenates were freshly prepared.

The activities of α-D and β-D glucosidase and α-D and β-D galactosidase were determined by the estimation of p-nitrophenol liberated by hydrolysis of the corresponding p-nitrophenyl glycoside[18]. The composition of the reaction mixtures was as follows:

Aryl α-D glucosidase 200 µl 0.1 M McIlvane's buffer, pH 5.8[19], 50 µl 51.3 mM p-nitrophenyl α-D glucopyranoside (to give a final concentration of 9.5 mM) and 20 µl homoge-

Table 1. Activation energies of oligosaccharidases present in the gut of *C. maculatus*

Enzyme	Temperature range (°C)	Activation energy (kJ · mole^{-1})
α-D-Glucosidase	18.0–57.2	35.80[a] 31.40[b]
α-D-Galactosidase	18.0–35.0	39.40[a] 38.30[b]
	35.0–51.0	7.85[a] 8.42[b]
β-D-Galactosidase	18.0–57.2	43.84[a] 38.30[b]

[a] and [b] represent independent experiments