with an equal volume of ethanol, suggesting that precipitation of protein with ethanol is dependant on the volume of the deproteinizing agent. The values are highly reproducible and reliable after deproteinizing with 20 volumes of ethanol.

For analysis at different times of day a haemolymph sample was collected every 2 h. The cut end was immediately cauterized. Analyses were carried out from 10.30 to 18.30 h at 2-h intervals. The total haemolymph protein concentration was determined using the Biuret method[22].

The haemolymph total calcium ranged from 26.67 to 49.59 mM/l. Bound calcium ranged from 27.84 to 43.35 mM/l. The free calcium ranged from 1.46 to 9.17 mM/l. Protein concentration ranged from 3.46 to 4.86 gm%.

Protein concentration and bound calcium fluctuated identically in that they increased to a maximum at 12.30 h and 14.30 h respectively and declined thereafter (table 2). This finding suggests that Ca in the haemolymph may be complexed predominantly with protein.

It is obvious that the increase in bound-Ca level is due to an increase in protein level. Whatever the source that liberates protein into the haemolymph, it is doing so in such a way that the protein is liberated in the form of calcium proteinate. At 14.30 h, when the protein level in the haemolymph remained constant, about 9.3 mM/l of bound Ca was added in the haemolymph and about 4 mM/l of free Ca disappeared from the haemolymph. Probably the free Ca complexed with the protein at 14.30 h. The proteins may accomodate free Ca by exposing anionic sites, for it is known that soluble proteins change their structure under the influence of pH[23].

The total amount of bound Ca in the haemolymph at 14.30 h is twice that of the free Ca that has disappeared at that hour. It is possible that free Ca from other tissues might have contributed to the increase in bound Ca. It is also likely that some fraction of the bound calcium may represent Ca bound with lipids[9-12] and acid mucopolysaccharides[13].

The fall in bound Ca and haemolymph protein concentration and corresponding increase in free Ca in the haemolymph after 14.30 h argue for the dissociation of Ca from its binding sites; this study stresses the need to investigate variation in the pH of the haemolymph at different times of day because association and dissociation of Ca with protein have been shown to be due to changes in pH[18,24].

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Insect hemocytes: Cells adapted to anaerobiosis

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Summary. Hemocytes from a free-living insect could be grown under strictly anoxic conditions. Their anaerobic metabolism was studied in vitro in such a cellular system.

In most species of insects, oxygen and carbon dioxide are exchanged directly between the cells and the atmosphere through a complex tracheal system. The hemolymph, devoid of respiratory pigment, does not ensure effective respiratory gas exchange, and its oxygen partial pressure and redox potential are low[2-4]. The hemocytes, farthest from the endings of the tracheae, are probably exposed to hypoxic or even anoxic conditions. Actually, the first successful primary cultures of these cells were obtained under a nitrogen atmosphere, containing 3% carbon dioxide and only 1% oxygen[5]. Established hemocyte lines from the American cockroach *Periplaneta americana* L. (HPa strains)[6] gave us the opportunity to characterize in vitro the anaerobic capacities of these cells.

HPa lines were isolated from embryos, nymphs or adults of *P. americana* in the culture medium S20 designed to reproduce the main physico-chemical characteristics of cockroach hemolymph[6]. The concentrations of amino acids and reducing components were high, especially glucose (22 mmoles $\cdot l^{-1}$) and cysteine (4 mmoles $\cdot l^{-1}$). Ascorbic acid (0.015 mmoles $\cdot l^{-1}$) and reduced glutathione (0.05 mmoles $\cdot l^{-1}$) were added.

Normoxia. Cell growth of hemocytes cultured in cotton-stoppered flasks was minimal and the pH of the culture medium rose from 7.4 to around 7.6 (table).

Progressive hypoxia. Cell growth was optimal in rubber-stoppered flasks with a limited initial oxygen store (90 μmoles $\cdot l^{-1}$ for 10^5 cells). In 7-day-old cultures, the oxygen

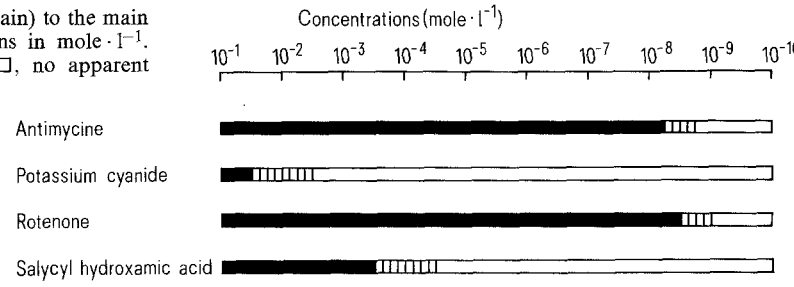

Fig. 1. Sensitivity of cockroach hemocytes (HPa strain) to the main inhibitors of the respiratory chain. Concentrations in mole · l^{-1}. ■, lethal effect; ▥, morphological effect; □, no apparent effect.

partial pressure fell from 154 to 80 torr. The HPa cells used up 2.4 mmoles · l^{-1} of glutamate or, in the absence of glutamate, 1.7 mmoles · l^{-1} of aspartate and released 1.7 or 1 mmoles · l^{-1} of alanine respectively. No lactate was detected, but appreciable amounts of succinate, acetate and propionate appeared. As a result, the pH of the culture medium dropped from 7.4 to 6.9–6.6. This acidosis can be partly related to carbon dioxide accumulation, since pH rose to 7.1–7.2 when the medium was equilibrated for 15 min against air.

Anoxia. The air in 3-day-old hemocyte cultures in rubber-stoppered flasks was replaced with pure nitrogen. 4 days later, acidosis increased sharply. This acidosis can be attributed, at least partly, to an increased concentration of acetate and propionate. Both succinate and alanine production decreased and the ratio alanine/acetate fell from 14 in hypoxia to 3 in anoxia.

HPa cells proved to be resistant to salycyl hydroxamic acid and cyanide (figure 1). In the presence of KCN (1 mmole · l^{-1}), the production of alanine and volatile fatty acids remained unchanged whereas succinate was no longer released into the medium (table).

These results show that insect hemocytes can be grown in vitro under progressive hypoxia and even under anoxia. In such conditions, they release by-products (alanine, succinate, acetate and propionate) typical of invertebrate anaerobic metabolism[7], which relies upon the simultaneous catabolism of carbohydrates and amino acids[8,9]. In the culture medium, as in insect hemolymph, amino acids as well as carbohydrates constitute an energy reserve. Their utilisation is initiated by important transamination reactions leading to ketoacids. Several amino acids are involved, but glutamate is the major substrate for alanine aminotransferase. This enzyme plays a key rôle in the anaerobiotic pathways and alanine accumulates as one of the main end products in vivo and in vitro[6].

In anoxic HPa cultures, glutamate is essential. Substitution of aspartate for glutamate, possible during hypoxia, proved to be unsuccessful in anoxia.

The bicarbonate ion also plays a major rôle in anaerobiosis. Indeed, it is required for the shift from phosphoenolpyruvate to oxaloacetate, which in turn is reduced to malate[10].

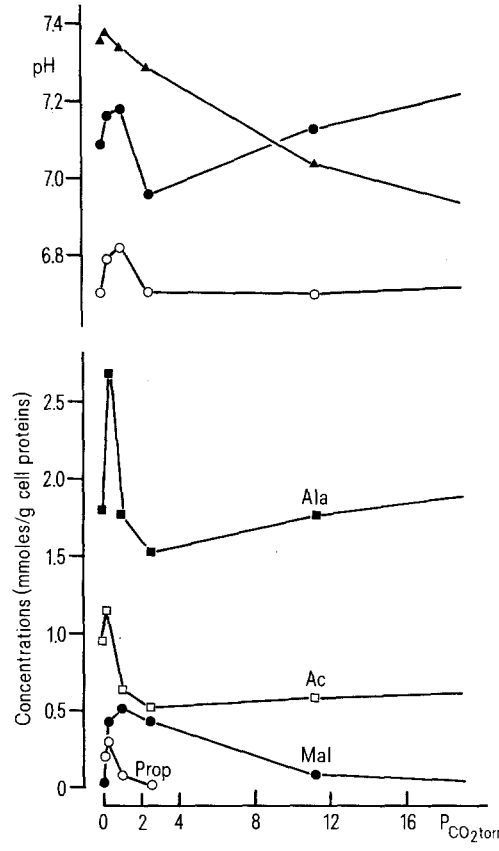

Fig. 2. Effects of carbon dioxide on anoxic cultures of cockroach hemocytes (mean data from 2 sets of experiments). Abscissa: carbon dioxide partial pressure in torr. Top: pH of the culture medium: —▲—, initial pH, —○—, after 7 days; —●—, 7-day culture medium equilibrated 15 min against air. Bottom: end-product concentrations in 7-day anoxic cultures. Ala, alanine. Ac, acetate. Mal, malate. Prop, propionate (× 10). Experimental conditions: 75-ml plastic flasks, culture area, 25 cm^2; Inoculum, 2.10^6 HPa cells; culture medium S20, 4 ml; temperature, 25 °C; gas phase, N_2 with variable percentage of CO_2 (0–3%).

Effects of culture conditions on cockroach hemocyte lines. Measured values of PO_2 (torr), pH and metabolic end products (mmole · l^{-1}) in 7-day culture medium (mean data from 3 sets of experiments. The results of preliminary investigations, performed to settle the experimental conditions described below were not taken into account)

	PO_2	pH	Acetate	Alanine	Lactate	Propionate	Succinate
Normoxia	153	7.6	0	-	0	0	-
Progressive hypoxia							
Without KCN	80	6.6–6.9	0.12	1.7	0	0.01	2.2
KCN (1 mmole · l^{-1})	95	6.7	0.15	1.6	0	0.01	0
Anoxia							
Normal cells	0	6.7	0.31	0.9	0	0.02	0.8
Vitamin B12-deficient cells	0	6.8	0.48	2.2	0	0	0.7

Experimental conditions: 11-ml glass flasks. Culture area, 10 cm^2. Inoculum, 10^5 HPa cells. Culture medium S20, 1.5 ml. Initial PO_2, 154. Initial pH, 7.4. Temperature, 25 °C. Gas phase, air.

We attempted to demonstrate that anoxic HPa lines require HCO_3^- (figure 2). Whatever the initial pressure of carbon dioxide in the nitrogen atmosphere (0–23 torr), the final acidosis of the culture medium was nearly the same, the proton concentration becoming stabilized at around 180 $neq \cdot l^{-1}$ (pH about 6.75). The release of end products reached maximal values for carbon dioxide pressures ranging between 0.2 and 1 torr. The cells failed to synthesize malate in the absence of HCO_3^- in the culture and they did not survive. Malate plays the rôle lactate does in vertebrates, but unlike lactate, it is not accumulated[8]. It easily enters the mitochondrion and there supports anaerobic production of ATP during its conversion to succinate. The generation of $NADH^+$ requires the linked dehydrogenation of the ketoacids produced by transamination. Mitochondrial particles isolated from parasitic worms catalyze an electron transport of this type[11]. Flavine protein and coenzyme Q are necessary, but it is not yet established whether a cytochrome b is involved. Cockroach hemocytes appeared to be very sensitive to rotenone and antimycine (figure 1), known to act as stoichiometric inhibitors of flavoproteins and cytochrome b respectively.

Nutritional requirements were easily characterized using anoxic cultures in chemically defined media. HPa cells need riboflavin and phenylalanine, both precursors of flavoproteins and coenzyme Q. Vitamin B12 is not required by the hemocytes under normoxia or hypoxia when nucleotide precursors are supplied, but becomes essential under anoxia. Cyanocobalamin-deficient cells begin to degenerate during the second week of culture: they fail to synthesize propionate, but release more acetate (table).

Thus, the biosynthetic pathways leading to propionate appear to be essential for cell survival. Among the by-products of anoxic metabolism, the less abundant, malate and propionate, are perhaps the most significant for the anaerobic production of energy.

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Presynaptic sympathetic supersensitivity following long-term preganglionic denervation

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Summary. After chronic preganglionic denervation of the rabbit ear artery, the contractile response to low frequency stimulation of the postganglionic innervation is increased. A preliminary analysis suggests that this cannot be accounted for by effector cell changes, but is due to an increase in adrenergic transmitter release.

Denervation supersensitivity of excitable tissues is a well documented occurrence[2,3]. We report here that long-term preganglionic denervation (decentralization) of adrenergic neurons results in increased responsiveness of the rabbit ear artery to electrical stimulation of sympathetic nerve terminals and to tyramine, an effect not accounted for by vascular smooth muscle hypersensitivity.

Unilateral preganglionic axotomy, by removal of about 3 cm of preganglionic nerve proximal to the superior cervical ganglion, under sterile conditions, was performed in young adult New Zealand white rabbits of 2.0–2.3 kg b.wt. 8 weeks after surgery the animals were sacrificed by a blow on the head and exsanguinated. Ring segments 3 mm long, dissected in situ from both the decentralized and control ear arteries were mounted in organ baths at 37.5 °C. Rings were suspended from stainless steel wires held in place by machined Lucite holders. One of the Lucite holders was stationary, the other was connected to a Statham G10b force-displacement transducer, which was coupled to a Grass polygraph chart recorder (model 79D) for displaying changes in vessel tension[4]. After equilibration for 1 h, the nerve terminals in the blood vessel wall were stimulated using biphasic pulses of 0.2 msec duration, at a supramaximal voltage from an electronic stimulator (Grass model S4). This mode of electrical stimulation, transmural nerve stimulation (TNS), elicited a response due solely to the release of norepinephrine (NE) from sympathetic nerve terminals[5], since it was abolished by tetrodotoxin (9×10^{-7} M).

The density and distribution of adrenergic nerve terminals, demonstrated by specific catecholamine fluorescence in the decentralized and control ear arteries, were similar. This contrasts with the disappearance of nerve terminals after postganglionic sympathetic denervation[6]. TNS caused frequency-dependent increases in contraction of the artery wall (figures 1 and 2). As seen in figure 1, a, the decentralized artery showed a contractile response to stimulation at 0.1 Hz. The phasic contractions seen in the figure correspond to each stimulus. In contrast, the threshold frequency for the control artery was 0.4 Hz (figure 1, b). The frequency-mean response curves of the decentralized and control ear arteries are shown in figure 2. At frequencies of up to 1.6 Hz, the contractile force of the decentralized artery was significantly greater than that of the control when compared using the paired t-test ($p < 0.05$). When the cells in a control artery were exposed to K^+ (10 mM), a twitch-like contraction was observed in response to TNS at 0.1 Hz in 2 out of 5 animals (figure 1, b). The concentration-response curve of the decentralized ear artery to tyramine, which is known to act by releasing catecholamines from sympathetic nerve terminals, was displaced to the left of the control artery curve. At the EC_{50} (concentration to elicit a response