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OXYGEN-CARRYING PERFLUOROCHEMICAL EMULSIONS IMPROVE INSECT FAT BODY CULTURE PERFORMANCE

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Abstract—The effect of an oxygen-carrying perfluorochemical emulsion (perfluorotri-*n*-butylamine) (PFE) on the oxygenation of larval *Manduca sexta* fat body culture was tested. Two different parameters, protein synthesis and secretion and ATP levels, were measured to evaluate the effect of PFE. After incubation for 21 h the ATP levels in fat body incubated in the presence of PFE were similar to those found *in vivo*; and significantly higher than in fat body incubated without PFE. The rate of protein synthesis and secretion increased with the concentration of PFE in the medium, up to a concentration of 20%. During incubation for 15 h a 10-fold higher rate of protein synthesis and secretion into the culture medium was found in the presence of PFE, compared to incubations without PFE. The synthesis and secretion of two major proteins produced by the fat body, arylphorin and high density lipophorin, was confirmed by immunoprecipitation. The results show that PFE improves the performance of cultured fat body tissue.

Key Word Index: *Manduca sexta*; fat body culture; perfluorochemical

INTRODUCTION

Development of culture media in which insect tissues maintain high levels of protein synthesis and secretion has been a difficult task. One of the main problems is maintenance of appropriate oxygenation of the cultured tissues. Several approaches have been used to maintain adequate oxygenation, such as keeping the tissue in an atmosphere rich in oxygen (Riddiford *et al.*, 1979), or maintaining the tracheal system intact and open (Kaatz *et al.*, 1983). We approached this problem by the use of an oxygen-carrying perfluorochemical emulsion (PFE). This emulsion shows a higher solubility for oxygen and carbon dioxide, as well as a higher diffusion coefficient for gases, compared to aqueous solutions at comparable partial pressures of gas (Clark and Golan, 1966; O'Brien *et al.*, 1982). PFE does not react with gases in solution, and the lack of toxicity has allowed its use as blood substitute for organ perfusion (Segel and Rendig, 1982; Chemnitius *et al.*, 1985).

We tested the effect of PFE on the ability of cultured fat body to maintain *in vivo* levels of ATP and to carry out protein synthesis and secretion; two processes depending on the energy status of the tissue.

MATERIALS AND METHODS

Insects

Manduca sexta were reared as described previously (Prasad *et al.*, 1986). Fifth instar larvae, 72 h post-ecdysis, were used in all the experiments.

Materials

Streptomycin sulfate and glutathione were obtained from Sigma (St Louis, Mo), gentamicin from JRH Bioscience (Lenexa, Kans.), [^{35}S]methionine from NEN (Boston, Mass.), Grace's medium from Gibco laboratories (Grand Island, N.Y.), trichloroacetic acid from Fisher (Fair Lawn, N.J.), Coomassie brilliant blue R-250 from Pierce (Rockford, Ill.), Amplify from Amersham (Arlington Heights, Ill.), Pansorbin from Calbiochem Co. (La Jolla, Calif.), and Falcon Multiwell tissue culture plates from Becton Dickinson Co. (Lincoln Park, N.J.).

Perfluorochemical

Oxypherol (Alpha therapeutic Corporation, Los Angeles, Calif.) is a sterile emulsion of 25% perfluorotri-*n*-butylamine in 3.2% polyoxyethylene-polyoxypropylene copolymer in water. Oxypherol is described as relatively stable when it is stored at 4°C. Our experience was that once opened, the solution must be used within 48 h in order to obtain good results. The pH of Grace's solution does not change when PFE is added. The viscosity of the solution at 25°C increases about 25% (Green Cross Co., 1980). The osmolarity of the solution is not affected by the presence of PFE.

After this work was finished, we learned that the manufacturer had discontinued production of oxypherol. We therefore prepared our own perfluorochemical emulsion by mixing 25% perfluorotri-*n*-butylamine (Aldrich, Milwaukee, Wisc.) and 3.2% Pluronic F-68 (polyoxyethylene-polyoxypropylene copolymer, Sigma) in water and emulsifying by several passes through a colloid mill. The emulsion was allowed to stand for 30 min to allow a small amount of insoluble material to settle out, and the supernatant was collected for use in the present study.

Dissection and incubation of fat bodies

Insects were surface-sterilized in 70% ethanol (Riddiford *et al.*, 1979). Fat bodies were dissected, halved longitudinally and rinsed three times with sterile phosphate-buffered saline (0.1 M sodium phosphate, pH 6.5, 0.15 M NaCl), containing 10 mM glutathione. Cultures were kept at room temperature (24°C) in 6 or 12 multiwell tissue culture plates containing 1 ml of Grace's medium with 100 μg/ml streptomycin, 50 μg/ml gentamicin and 10 mM glutathione. [^{35}S]methionine was

added to the media (100 μCi/ml). The medium was oxygenated by aerating with 95% O_2–5% CO_2 for 30 min before the beginning of the incubation, and then the incubation was carried out under normal air. The plates were agitated at 70 rpm during the incubation on an Orbital shaker (VWR, San Francisco, Calif.). In some experiments 95% O_2–5% CO_2 was gently blown over the medium. Incubations were terminated by freezing the fat bodies and media, which were stored at −70°C.

Protein precipitation

Total proteins were precipitated from 200 μl samples of medium by adding 50 μg of bovine serum albumin and trichloroacetic acid (TCA) to make a final TCA concentration of 10%. Samples were left at 4°C for 30 min. Precipitates were collected by centrifugation (15 min, 13,000 rpm) and washed twice with cold 5% TCA. Pellets were dissolved in 100 μl of 0.1 N NaOH and counted by liquid scintillation.

ATP assay

After incubation or dissection, samples were frozen (−70°C). The frozen tissues were homogenized using a Polytronal homogenizer (Brinkman Instruments Inc., Westbury, N.Y.) in 3.5 vol of 8% $HClO_4$ (v/v) in 40% ethanol (v/v) for 2 min (Williamson and Corkey, 1969). After centrifugation (10 min, 13,000 rpm) the pellet was re-extracted with the perchloric acid-ethanol solution. Supernatants were pooled and neutralized by adding 3 M K_2CO_3. The neutralized solution was diluted with 40 mM Hepes buffer of pH 7.5, and assayed by liquid scintillation counting using a bioluminescence assay (Boehringer Mannheim Biochemica).

Immunoprecipitation

Immunoprecipitation was performed using Pansorbin, as described by Ryan *et al.* (1988). Antibodies against arylphorin were raised as described by Ryan *et al.* (1985); and against high density lipophorin as described by Shapiro *et al.* (1984). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on samples of culture medium or immunoprecipitates by the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue R-250, and ^{35}S-labeled proteins were visualized by fluorography after soaking the gel in Amplify, drying *in vacuo*, and exposing to Kodak X-Omat AR film.

RESULTS AND DISCUSSION

ATP levels can be used as a measure of normal oxygenation of the tissue. The ATP-ADP cycle is the fundamental mode of energy exchange in biological systems; the turnover of ATP is very high, and under physiological conditions cells maintain a high level of ATP. A 2-fold increase in ATP levels was found in a rabbit heart preparation, when PFE was added to the perfusion medium (Freeman *et al.*, 1987). The ATP levels in fat body incubated with PFE for 21 h were comparable to those in freshly excised fat body, whereas the ATP level in fat body incubated without PFE was <2% of the *in vivo* value (Fig. 1).

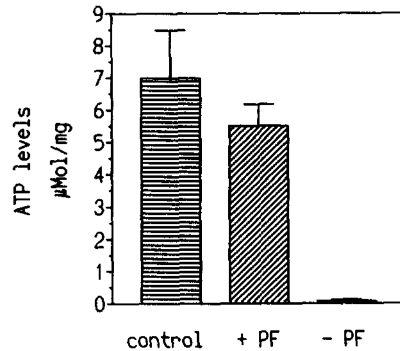

Fig. 1. ATP levels in fat body. After acid extraction, ATP was assayed using a bioluminescence assay. Control: the tissues were frozen immediately after dissection; +PF: fat body incubated for 21 h in Grace's medium with perfluorochemical emulsion: −PF: fat body incubated for 21 h in Grace's medium without perfluorochemical emulsion. Bars represent averages ±SD ($n = 3$). There was not a significant difference between control and +PF ($P > 0.1$); −PF was significantly less than either control or +PF ($P < 0.01$ in each case).

Since one of the major functions of insect fat body is the synthesis and secretion of hemolymph proteins, this highly energy-dependent process was used as a second parameter to analyze the effect of PFE on the performance of fat body in culture. Fat bodies were incubated for different lengths of time, in the absence or presence of different concentrations of PFE. The effect of increased concentrations of PFE in the medium on protein synthesis and secretion is shown in Fig. 2. It was found that there was a concentration-dependent increase in protein synthesis and secretion in media containing PFE, and that protein synthesis and secretion in media with PFE was 5-fold higher than in media without PFE. In subsequent experiments PFE was used at a concentration of 20%. At this concentration no abnormal morphological changes in the incubated tissues were detected under the light microscope (data not shown).

Arylphorin and high density lipophorin are two major hemolymph proteins synthesized and secreted by larval fat body (Kanost *et al.*, 1990). By immunoprecipitating the incubation media with specific antibodies, the secretion of these proteins in the presence of PFE was confirmed (Fig. 3).

There was a linear increase in protein synthesis and secretion into the incubation medium in both the presence and absence of PFE (Fig. 4); however, the rate was 10-fold higher in the presence of PFE (3450 cpm/h) than in its absence (350 cpm/h). In order to analyze the viability of the tissues after a

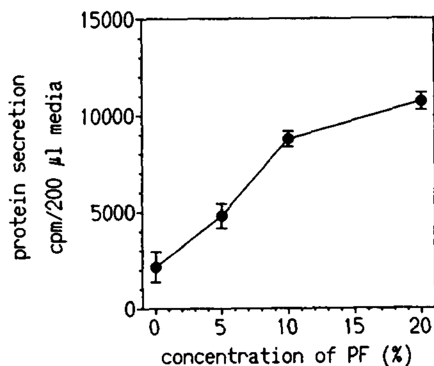

Fig. 2. Effect of increasing concentrations of perfluorochemical emulsion on protein synthesis and secretion. Fat bodies were incubated for 15 h in Grace's medium containing 100 μCi [^{35}S]methionine. Labelled proteins in the media were determined by TCA precipitation and liquid scintillation counting. Points represent averages ±SD ($n = 4$). P, 20% PFE vs no PFE <0.01.

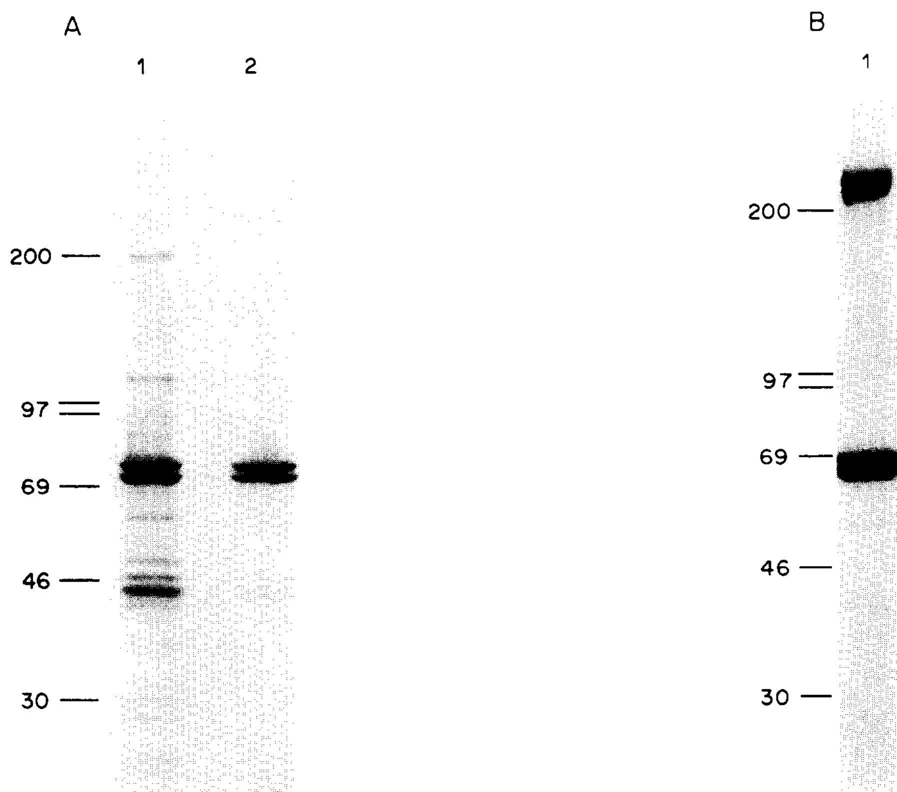

Fig. 3. Immunoprecipitation of the labeled proteins from Grace's medium in the presence of perfluorochemical emulsion. Fat bodies were incubated in Grace's medium with PFE for 21 h, and then in Grace's medium containing 100 μCi of [^{35}S]methionine instead of methionine for 45 min. Immunoprecipitations were performed using Pansorbin. Immunoprecipitates were subjected to SDS–PAGE. Panel A: 1 = total labeled proteins; 2 = proteins immunoprecipitated with arylphorin antibody. Panel B: 1 = proteins immunoprecipitated with high density lipophorin antibody. The position of molecular weight markers (kDa) are shown by lines.

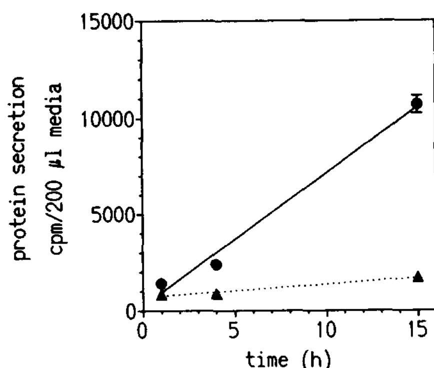

Fig. 4. Protein synthesis and secretion as a function of time of incubation. Fat bodies were incubated in Grace's medium for different lengths of time, in the presence or absence of PFE, containing 100 μCi [^{35}S]methionine. Labeled proteins in the media were determined by TCA precipitation and liquid scintillation counting. ●: Grace's medium with 20% PFE; ▲: Grace's medium alone. Points represent averages $\pm$SD (within the symbol, if not shown) ($n = 4$). There was significantly more protein secreted in the presence of PFE (at 1 h $P < 0.05$; at 4 and 15 h $P < 0.01$).

long incubation period, fat bodies were preincubated in Grace's medium with or without PFE for 21 h, and then transferred to 1 ml of Grace's medium containing [^{35}S]methionine in place of methionine, and incubated for 45 min. Fat body which had been preincubated in media containing PFE produced a 7.5-fold larger amount of TCA-precipitated proteins than fat body that had been preincubated without PFE (Fig. 5). This result shows that even after incubation for 21 h in PFE media fat body was still healthy and capable of high levels of protein synthesis.

Oxygen solubility in PFE emulsions changes linearly with the pO_2, according to Henry's law (Green Cross Co., 1980), and it was assumed to be possible that the incubation medium lost significant oxygen during the incubation for 21 h. We tested this possibility, by continuously blowing a 95% O_2–5% CO_2 mixture over the culture container during incubation for 21 h.

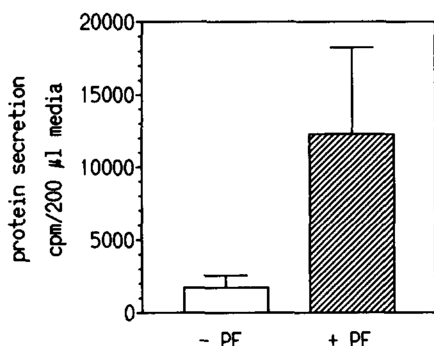

Fig. 5. Protein synthesis and secretion after 21 h of incubation. Fat bodies were incubated as described in Fig. 3. Labeled proteins in the media were determined by TCA precipitation and liquid scintillation counting. Open bar = Grace's medium; hatched bar = Grace's medium with 20% PFE. Bars represent averages $\pm$SD ($n = 9$). There was significantly more protein secretion in the presence of PFE ($P < 0.01$).

A small, but not significant, increase in the amount of secreted proteins was observed under these conditions (data not shown); therefore, it is sufficient to oxygenate the PFE solutions for 30 min before incubation in order to ensure good oxygen levels for 21 h.

All experiments reported to this point were carried out using oxypherol. When we learned that the manufacturer had discontinued production of oxypherol, we prepared our own emulsion, as detailed in Materials and Methods, and tested its ability to support protein synthesis and secretion. There was no difference between the laboratory-prepared emulsion and oxypherol in an experiment in which protein synthesis and secretion was measured for 15 h (data not shown). We concluded that a laboratory-prepared emulsion is as effective as the commercial product.

Based on the results reported here, it is concluded that PFE can improve oxygenation of *in vitro* fat body cultures, resulting in higher ATP levels, and higher rates of protein synthesis and secretion. The results demonstrate the advantage of using PFE for incubation of insect tissues.

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