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IMPROVED CONDITIONS FOR CULTURE OF BIOSYNTHETICALLY ACTIVE COCKROACH CORPORA ALLATA

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SUMMARY

Currently, short-term culture of insect corpora allata is most often performed in TC199. We now show that L-15B, a medium widely used in arthropod tissue culture, is superior to TC199 for both short- and long-term culture of cockroach corpora allata. In 3-h and 48-h incubations, juvenile hormone biosynthesis by corpora allata from *Diploptera punctata* was significantly higher in L-15B than in TC199. In addition, in both media, corpora allata activity was significantly improved by flotation of glands at the medium surface. Characteristics of L-15B responsible for its superiority were examined by comparison of gland activities in several TC199 formulations that had been modified in different ways to be more similar to L-15B. Adjusting the osmotic pressure of TC199 (288 mOsm/l) to near that of L-15B (362 mOsm/l) and *D. punctata* hemolymph (360 mOsm/l) significantly improved gland activity during the second 12 h of a 36-h incubation. Increasing the concentrations of amino acids, sugars, and organic acids in TC199 to the same levels as in L-15B significantly improved gland activity during both the second and third 12-h intervals of a 36-h incubation. These results suggest that L-15B is superior to TC199 because L-15B is isoosmotic with *D. punctata* hemolymph and because L-15B, like cockroach hemolymph, contains a high level of organic constituents. It is therefore more appropriate to use L-15B than TC199 for short-term *in vitro* assays of juvenile hormone biosynthesis and for extended corpora allata culture.

Key words: corpora allata; juvenile hormone; cockroach; *in vitro* radiochemical assay; insect organ culture; *Diploptera punctata*.

INTRODUCTION

The culture *in vitro* of insect corpora allata (CA) is a valuable tool for understanding the function of these organs *in vivo*. Of great importance to studies on CA endocrinology has been the development of a radiochemical assay for measurement of juvenile hormone (JH) biosynthesis by CA *in vitro* (Pratt and Tobe, 1974). Widespread use of this assay has enabled elucidation of biochemical pathways in JH biosynthesis (Schooley and Baker, 1985) and has promoted understanding of neural, neuropeptide, and dietary regulation of JH production (Tobe and Stay, 1985; Schal et al., 1993; Stay et al., 1994). Moreover, studies on insect reproduction have benefited from investigators' ability to correlate CA activity with oocyte maturation (Feyereisen, 1985).

Primarily three culture media, Grace's medium, minimal essential medium (MEM) and medium 199 (TC199), have been used in the radiochemical assay, and CA have been shown to produce JH at high rates in these media in short-term incubations. Whereas Grace's medium is used mainly with lepidopteran CA (Granger et al., 1986), MEM has been used with CA from diverse insects, including *Locusta migratoria* (Gadot and Applebaum, 1985) and *Drosophila melanogaster* (Richard et al., 1989). Overall, however, TC199 has been the most extensively used medium in radiochemical assays and is used with CA from numerous species of Orthoptera, Blattaria, Isoptera, Coleoptera, Lepidoptera, Diptera, and Hymenoptera (Kramer and Law, 1980; Tobe and Stay, 1985; Zou et al., 1989; Cusson et al., 1990; Borovsky and Carlson, 1992).

Despite widespread use of TC199 in radiochemical assays, several studies indicate that this medium is suboptimal. Whereas low activity CA of *Schistocerca gregaria* release JH at constant rates for up to 5 h in TC199, release rates from CA with intermediate to high activity decline rapidly after only 3 h (Pratt et al., 1975). A similar pattern of declining gland activity occurs in assays of CA from *Leptinotarsa decemlineata*, *Tenebrio molitor*, and *Phormia regina* (Weaver et al., 1980; Khan et al., 1982; Zou et al., 1989). Finally, JH release rates of moderately to highly active CA from the cockroaches *Periplaneta americana*, *Nauphoeta cinerea*, and *Blattella germanica* diminish after 8 to 10 h in TC199 (Pratt et al., 1976; Lanzrein et al., 1978; Bellés et al., 1987).

The inability of TC199 to sustain constant CA activity in long-term incubations could be due to the low nutrient concentration and osmotic pressure of TC 199 compared with insect hemolymph and standard insect cell culture media. Because successful culture of insect cells often requires use of isoosmotic nutrient-rich media (Grace, 1982), we speculated that *in vitro* activity of *Diploptera punctata* CA would be improved by incubating glands in L-15B, a medium with a high level of organic constituents and an osmotic pressure similar to that of *D. punctata* hemolymph. Our results show that in both short- and long-term assays, JH biosynthesis by *D. punctata* CA is significantly higher in L-15B than in TC199.

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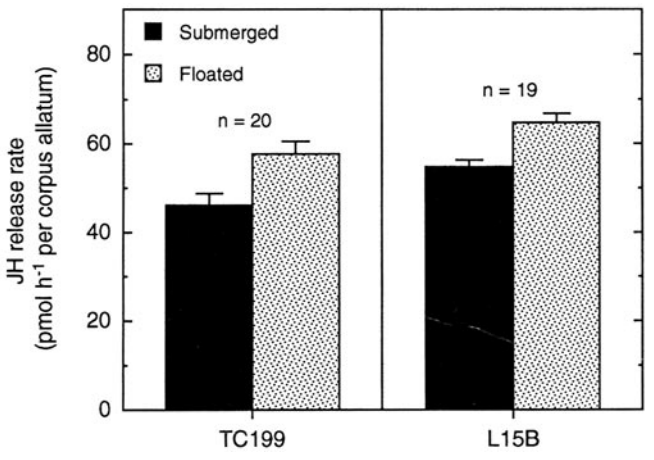

FIG. 1. Effect of flotation versus submersion on CA activity in TC199 and L-15B. Gland pairs were split and one gland from each pair was floated at the medium surface while the contralateral gland was submerged beneath the medium surface in a different culture tube. Both glands of a pair were incubated in the same type of medium for 3 h. *Bars* represent means + SEM.

MATERIALS AND METHODS

Insects. The *D. punctata* colony was maintained at 27 ± 0.3° C under a 12 h light:12 h dark photoperiod and was supplied with Purina rat chow and water *ad libitum*. Newly eclosed adult females were mated and kept thereafter in groups of 10–20 under conditions similar to those of the colony.

Incubation media. TC199 without methionine and calcium and with L-glutamine, Hanks' salts and 25 m*M* HEPES was obtained as a special formulation from GIBCO (Grand Island, NY). L-15 was formulated without methionine by Specialty Media (Lavallette, NJ). L-15B was prepared from methionine-free L-15 as described by Munderloh and Kurtti (1989). TC199 and L-15B were supplemented with 20 mg Ficoll (type 400)/ml, and for both media $CaCl_2$ concentration was adjusted to 5 m*M* (Kikukawa et al., 1987) and pH was set at 7.2. In experiments calling for supplementation of TC199 with 34 m*M* amino acids, the following amounts and types of amino acids were added to 100 ml TC199: 40 mg DL-alanine, 44 mg L-arginine (free base), 25 mg L-asparagine (anhydrous), 23 mg L-aspartic acid, 11 mg L-cysteine (free base), 50 mg L-glutamine, 37 mg L-glutamic acid, 15 mg glycine (free base), 23 mg L-histidine (free base), 29 mg L-proline, 21 mg DL-isoleucine, 0.5 mg L-leucine, 2 mg L-lysine, 20 mg DL-phenylalanine, 15 mg L-serine, 54 mg DL-threonine, 26 mg L-tyrosine (free base), and 15 mg DL-valine. All modifications were performed with "cell culture tested" chemicals obtained from the Sigma Chemical Company (St. Louis, MO). Medium and hemolymph osmotic pressures were determined with a freeze-point osmometer (Advanced Instruments, Littleton, CO, Micro-osmometer 3MO).

Dissections. Highly active CA were obtained from 4-d-old mated females with oocyte lengths of 1.15 to 1.54 mm (Feyereisen et al., 1981). Females were briefly anesthetized with CO_2 and decapitated. Gland pairs were dissected from heads beneath cockroach saline (Kurtti and Brooks, 1976) modified to 360 mOsm/l, the osmotic pressure of *D. punctata* hemolymph.

Measurement of CA activity. JH release by CA *in vitro* was measured with a rapid partition radiochemical assay (Feyereisen and Tobe, 1981). This assay is based upon stoichiometric incorporation of a radiolabeled methyl group from methionine into JH under equilibrium conditions. Before all assays, CA were preincubated for at least 45 min in either L-15B or TC199 containing 100 μ*M* L-[methyl-^{3}H]methionine (198 mCi/mmol; New England Nuclear, Wilmington, DE) in order to equilibrate radiolabeled exogenous methionine with intraglandular methionine. After preincubation, glands were transferred into 6 × 50 mm borosilicate glass culture tubes containing 100 μl of the same type of radiolabeled medium in which glands had been preincubated. Assay tubes with CA were incubated at 27° C and were subjected to wave action shaking on a rotating variable plane mixer (Waver, VWR) at 90 rpm and at a 16° pitch. At termination of an assay, CA were removed and medium was extracted with 250 μl isooctane. We determined JH release by assaying an aliquot of the isooctane hyperphase in a liquid scintillation spectrometer. We corrected measurements by subtracting radioactivity in the isooctane phase from blank incubations without CA.

Statistics. Paired-sample and two-sample *t*-tests were one-tailed, except where noted. Standard error of the mean (SEM) was used as the measure of variability.

RESULTS

Effect of surface incubation on JH production. In a previous effort to establish long-term culture conditions for locust CA, we embedded CA in agar-solidified L-15B so that glands were directly exposed to atmospheric oxygen (Gadot et al., 1993). To optimize culture conditions in liquid L-15B, we examined the effect of gland flotation on JH biosynthesis. CA pairs were split and one gland from each pair was randomly selected to be floated at the medium–air interface in a culture tube while the contralateral gland was gently submerged beneath the medium surface to the bottom of a different culture tube. In both TC199 and L-15B, floated glands produced significantly more JH than contralateral submerged glands (Fig. 1; $P < 0.005$ for TC199, $P < 5 \times 10^{-6}$ for L-15B; paired *t*-tests). CA were floated in all subsequent experiments.

The split-gland design used in Fig. 1 was based on the assumption that members of a gland pair produce similar amounts of JH when cultured under the same conditions. To test this assumption we dissected 13 gland pairs and incubated the two glands of each pair in separate culture tubes containing L-15B. In a 3-h assay, the JH release rate of right-hand glands (62.9 ± 3.7 pmol JH h^{-1}) was nearly identical ($P = 0.83$, two-tailed paired *t*-test) to that of left-hand glands (63.6 ± 3.9 pmol JH h^{-1}).

Methionine concentration. Before comparing CA activity in TC199 and L-15B it was necessary to identify an optimal methionine concentration for use in the radiochemical assay. Gland pairs were incubated in L-15B containing L-[methyl-^{3}H]methionine (L-met) concentrations ranging from 20 μ*M* to 120 μ*M*. Between 20 μ*M* and 80 μ*M* L-met, JH release rates increased linearly from 31.8 to 126.7 pmol h^{-1} (Fig. 2). JH release rates plateaued at or above 80 μ*M* L-

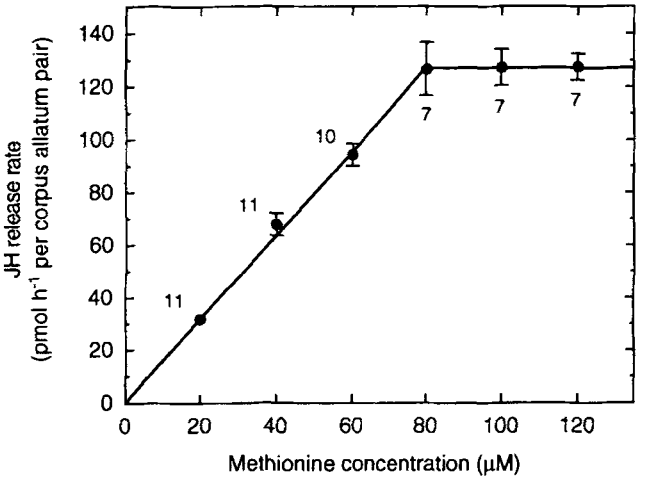

FIG. 2. Dose-response relationship for JH release as a function of methionine concentration. CA were incubated in L-15B for 3 h. Each point represents mean ± SEM with the number of individual measurements shown beside each point.

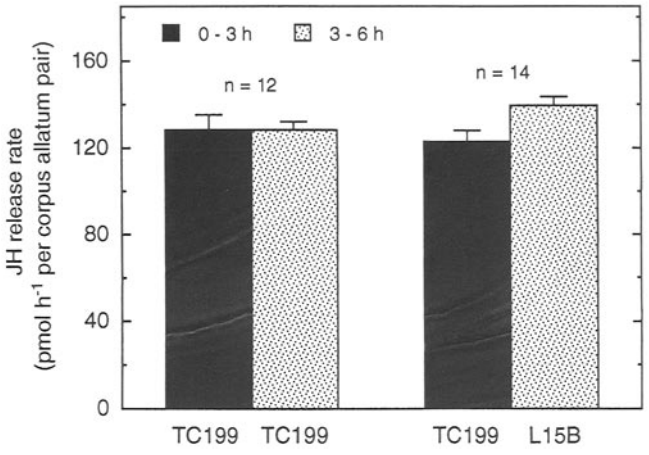

FIG. 3. Effect of L-15B on JH production by CA during the second 3 h of a 6 h incubation. CA were incubated for 3 h in TC199 and were then transferred to either TC199 or L-15B for an additional 3 h. *Bars* + SEM show mean rates of JH release during the first and second 3-h intervals. All glands were preincubated in TC199, and the medium in which CA were incubated during each 3-h interval is indicated beneath each *bar*.

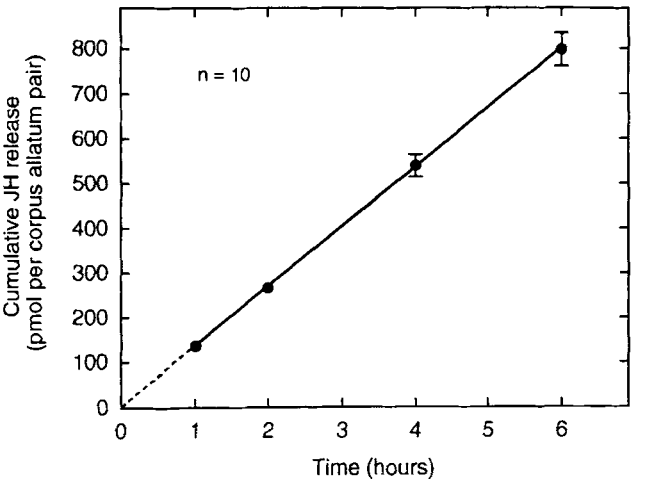

FIG. 4. Cumulative JH release in L-15B. CA were preincubated for at least 45 min in L-15B with radiolabeled methionine and were then transferred to new medium for the radiochemical assay. After 1 h, CA were again transferred to new medium, and the medium in which glands had been incubating was extracted to determine JH release during the 1-h interval. This procedure was repeated after an additional hour and thereafter at 2-h intervals. The assay was terminated after 6 h. Each point represents mean ± SEM of 10 measurements.

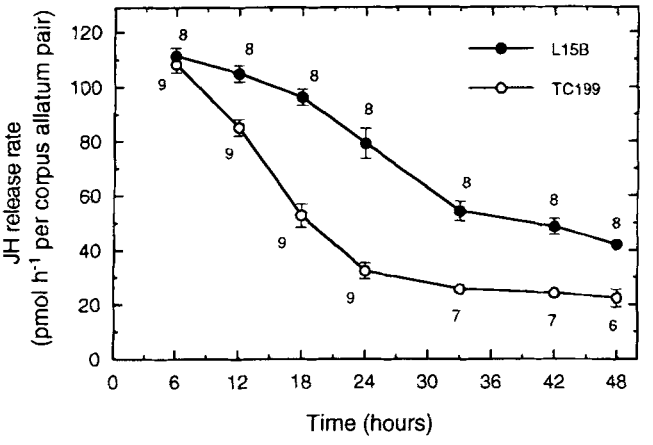

FIG. 5. JH production in long-term incubations. CA were incubated in either TC199 or L-15B for 48 h. At 6- or 8-h intervals, CA were transferred to new medium, and old medium was extracted to determine JH release. Three gland pairs in TC199 were removed from the experiment after they had sunk beneath the medium surface. Values are means ± SEM for the number of individual measurements shown beside each data point.

met. Because 100 μ*M* L-met was sufficient for highly active CA to attain maximal JH release rates, this concentration was used in all radiochemical assays, except where noted.

Comparison of media in short-term assays. A sequential assay was used to compare CA activity in L-15B and TC199. Gland pairs were incubated for 3 h in TC199 and were then transferred to either TC199 or L-15B for an additional 3 h of incubation. CA that remained in TC199 throughout the assay showed similar ($P = 0.48$, paired *t*-test) rates of JH release during the first (128.5 ± 6.8 pmol h^{-1}) and second (128.2 ± 4.0 pmol h^{-1}) 3-h intervals (Fig. 3). Gland pairs switched to L-15B after 3 h in TC199 produced 13.4% more JH ($P < 5 \times 10^{-4}$) during the second 3-h interval (139.5 ± 4.0 pmol h^{-1}) than during the first (123.0 ± 5.1 pmol h^{-1}).

Split-gland experiments confirmed results from sequential assays. When individual glands of a pair were incubated in different media at 27° C, glands in L-15B (67.3 ± 1.4 pmol h^{-1}) released significantly more ($P < 0.005$, paired *t*-test) JH than glands in TC199 (59.6 ± 1.9 pmol h^{-1}). To increase glandular metabolism and demand upon the medium, incubation temperature was raised to 30° C. At this temperature, JH release rates increased in both media (63.8 ± 3.6 pmol h^{-1} in TC199, 76.8 ± 3.5 pmol h^{-1} in L-15B), but glands still produced significantly more JH in L-15B ($P < 0.005$). Furthermore, at 30° C the magnitude of the difference between means became greater than at 27° C, increasing from 7.7 pmol h^{-1} (12.9% of the mean for TC199) to 13.0 pmol h^{-1} (20.4% of the mean for TC199).

Linearity of JH release. The suitability of a medium for short-term radiochemical assays depends on the ability of the medium to support constant rates of JH biosynthesis (Tobe and Stay, 1985). We examined whether L-15B met this criterion and found that JH release by 10 gland pairs was constant throughout a 6-h incubation (Fig. 4).

Comparison of media in long-term incubations. Results from short-term assays prompted us to explore the relative utilities of L-15B and TC199 in long-term CA culture. In both media, gland pairs released JH throughout a 48-h incubation, but the pattern of JH release differed between the two media (Fig. 5). Whereas JH release rates in L-15B declined only slightly from 111.4 ± 2.9 pmol h^{-1} after 6 h to 105.0 ± 3.0 pmol h^{-1} after 12 h, CA activity in TC199 fell by almost 20% from 108.4 ± 3.1 pmol JH h^{-1} to 85.2 ± 3.0 pmol JH h^{-1}. At 24 h, CA activity was substantially higher in L-15B (79.2 ± 5.6 pmol JH h^{-1}) than in TC199 (32.4 ± 2.9 pmol JH h^{-1}). After 24 h, JH release rates were 22–25 pmol h^{-1} in TC199 but never below 40 pmol h^{-1} in L-15B. Overall, in 48 h CA in L-15B released 3.53 ± 0.11 nmol JH, whereas those in TC199 released only 2.28 ± 0.16 nmol.

TABLE 1

EFFECTS OF MODIFICATIONS TO TC199 ON JUVENILE HORMONE BIOSYNTHESIS

Formulation[a]	Number	Osmolality (mOsm/l)	Juvenile Hormone Release[b] 0–12 h	12–24 h	24–36 h
TC199	12	288	120.9 ± 5.2	76.5 ± 4.0 (63.5 ± 2.9[c])	36.5 ± 2.6 (30.8 ± 2.7[c])
TC199	18	366	112.7 ± 2.9	89.8 ± 3.7 (79.5 ± 2.2[a,c])	39.9 ± 2.8 (35.5 ± 2.3[a])
+ 5 mM pyruvic acid and 2 mM α-ketoglutaric acid	16	359	112.1 ± 4.0	90.4 ± 4.3 (80.6 ± 2.5[a,c])	46.5 ± 3.0 (41.8 ± 2.7[a,c])
+ 5 mM D-galactose and 6.9 mM D-glucose	11	359	112.4 ± 4.6	93.1 ± 4.6 (82.9 ± 2.4[a])	49.2 ± 3.8 (44.4 ± 3.6[a,b,c])
+ 34 mM amino acids	11	365	121.5 ± 6.6	97.9 ± 6.0 (80.7 ± 3.1[a,c])	62.9 ± 3.7 (53.2 ± 4.1[a,b])
+ All above components	15	364	121.2 ± 3.1	97.1 ± 2.9 (80.5 ± 2.3[a,c])	63.1 ± 3.0 (52.4 ± 2.5[a,b])
+ 34 mM D-glucose	10	366	91.8 ± 3.0	63.5 ± 3.7 (69.0 ± 3.4[c])	35.7 ± 2.8 (38.9 ± 2.9[c])
+ 34 mM D-trehalose	9	367	117.3 ± 3.4	99.6 ± 6.2 (84.5 ± 3.8[a])	59.6 ± 4.6 (50.7 ± 3.5[a,b])
L-15B	16	362	127.7 ± 3.6	108.1 ± 3.0 (85.0 ± 1.8[a])	69.6 ± 2.8 (55.1 ± 2.5[a])

[a]Unmodified TC199 lacks galactose, pyruvic acid and α-ketoglutaric acid but contains 5.5 m*M* glucose and about 8 m*M* total amino acids. L-15B contains 5 m*M* galactose, 12.4 m*M* glucose, 5 m*M* pyruvic acid, 2 m*M* α-ketoglutaric acid and about 42 m*M* total amino acids. For all modified TC199 formulations, sufficient NaCl was added to raise osmotic pressure to near 360 mOsm/l.

[b]CA from females with oocyte lengths of 1.25–1.54 mm were incubated for three consecutive 12-h intervals in unmodified TC199, TC199 modified as indicated, or L-15B. Mean JH release rates expressed in pmol h^{-1} per gland pair are shown for each 12-h interval. The mean percent of initial activity retained by CA during the second and third incubation intervals is shown in parentheses. We determined percent values for each gland pair by dividing JH release during the second and third 12-h intervals by JH release during the first 12-h interval. Means within each incubation interval followed by different letters are significantly different from each other ($P < 0.05$, ANOVA, Games-Howell multiple comparison of means test, arcsin transformation of proportions).

[c]Retention of CA activity between 12 and 24 h was significantly lower by CA in TC199 at 288 mOsm/l than by CA in TC199 modified to 366 nOsm/l with NaCl ($P < 5 \times 10^{-4}$, two-sample *t*-test, arcsin transformation of proportions).

Modification of TC199. To gain insight into potential modifications of L-15B for improved CA culture, we identified characteristics of L-15B making it superior to TC199. TC199 was modified in several ways to be more similar in composition to L-15B, and JH release rates in different TC199 formulations were compared in 36-h incubations. In unmodified TC199 at 288 mOsm/l, the rate of JH release dropped to 63.5% of its initial value between 12 and 24 h and to 30.8% between 24 and 36 h (Table 1). Raising the osmotic pressure of TC199 with NaCl to 366 mOsm/l, an osmotic pressure similar to that of L-15B and *D. punctata* hemolymph, significantly improved gland activity ($P < 5 \times 10^{-4}$, two-sample *t*-test, arcsin transformation of proportions) during the second 12-h interval. Between 24 and 36 h, however, CA activity was no better in TC199 at 366 mOsm/l than in unmodified TC199 ($P = 0.11$). All subsequent modifications of TC199 were accompanied by adjustment of osmotic pressure to near 360 mOsm/l.

We next examined the effect of increasing the concentrations of amino acids, sugars and organic acids in TC199 to the same levels as in L-15B. In TC199 containing the same amounts of pyruvic acid and α-ketoglutaric acid or glucose and galactose as in L-15B, gland pairs retained more than 40% of initial activity between 24 and 36 h but did not retain significantly more activity than glands in TC199 modified to 366 mOsm/l with NaCl (Table 1). CA activity was significantly improved ($P < 0.05$, analysis of variance, Games–Howell multiple comparison of means) between 24 and 36 h by augmenting TC199 with 34 m*M* various amino acids, bringing the concentrations of these amino acids to the same levels as in L-15B. A similar result was obtained with 34 m*M* trehalose but not with 34 m*M* glucose which greatly depressed CA activity during each 12-h interval. Retention of gland activity was similar in TC199 augmented with 34 m*M* amino acids or 34 m*M* trehalose and in TC199 modified to contain the same types and amounts of amino acids, sugars, α-ketoglutaric acid, and pyruvic acid found in L-15B. Overall, between 12 and 36 h, CA in L-15B maintained proportionally, yet not significantly, higher rates of JH production than CA in any of the TC199 formulations (Table 1).

Modification of L-15B. Because high glucose levels inhibited JH biosynthesis whereas high trehalose levels promoted it (Table 1), we examined whether CA activity would be improved by incubating glands in L-15B containing only 1 m*M* glucose, reduced from 12.4 m*M*, and 34 m*M* trehalose. During the third 12-h interval of a 36-h assay, CA in modified L-15B retained slightly, but not significantly, higher activity ($P = 0.15$ and $P = 0.09$ for the 1.15–1.24 mm and 1.25–1.34 mm oocyte length categories, two-sample *t*-test, arcsin transformation of proportions) than CA in L-15B (Table 2). In a 3-h split-gland assay, the rate of JH release by individual glands in modified L-15B (68.8 ± 3.5 pmol h^{-1}) was similar ($P = 0.35$, paired *t*-test, n = 15) to that by contralateral glands (67.7 ± 3.3 pmol h^{-1}) in L-15B.

Discussion

Establishment of incubation conditions. Insect organ function *in vitro* is often improved when methods are used to promote tissue oxygenation. For example, ecdysteroid production by cultured prothoracic glands is significantly enhanced by elevation of atmospheric oxygen tension (Chino et al., 1974). The necessity of copious oxygen for optimal CA function was questioned in a previous unsuccessful attempt to improve activity of *S. gregaria* CA by culturing glands under high oxygen levels (Tobe and Pratt, 1974). Whether glands were floated or submerged in this study was not reported, but if glands were floated, then they may have received ample oxygen under normal atmosphere to support maximal gland activity. On the other hand, if CA were submerged, they may not have benefited from higher

TABLE 2

EFFECT OF TREHALOSE AND REDUCED GLUCOSE ON JUVENILE HORMONE BIOSYNTHESIS

Oocyte Length[a] Medium Formulation	Number	Juvenile Hormone Release[b] 0–12 h	12–24 h	24–36 h
1.15–1.24 mm				
L-15B	10	105.2 ± 5.0	99.2 ± 7.2 (93.6 ± 3.0)	79.3 ± 7.9 (74.1 ± 4.4)
Modified L-15B	8	119.1 ± 8.7	116.8 ± 9.5 (97.6 ± 1.7)	96.5 ± 8.1 (81.0 ± 3.1)
1.25–1.34 mm				
L-15B	7	123.2 ± 4.6	109.6 ± 6.9 (88.8 ± 4.3)	83.0 ± 6.7 (67.5 ± 5.3)
Modified L-15B	10	129.2 ± 5.5	114.9 ± 4.5 (89.1 ± 1.2)	97.5 ± 5.0 (75.3 ± 1.9)

[a]CA from adult females with oocyte lengths of 1.15–1.34 mm were incubated for three consecutive 12-h periods in L-15B or modified L-15B containing 34 m*M* trehalose and only 1 m*M* glucose. The L-methionine concentration in both media was 1 m*M* with radiolabeled L-methionine comprising 100 μ*M* of that amount.

[b]JH release rates expressed in pmol h^{-1} per gland pair are shown for each 12-h interval. The percent of initial activity retained by CA during the second and third incubation intervals is shown in parentheses.

oxygen levels. We are currently investigating the role of oxygen in maintenance of CA function.

The level of JH biosynthesis by CA *in vitro* depends upon the concentration of methionine in the medium (Tobe and Clarke, 1985). In the absence of methionine, CA produce little JH, but rates of JH biosynthesis rise linearly as methionine concentration is increased to a threshold. We have found that 80 μ*M* L-met is the minimal concentration necessary for *D. punctata* CA to attain maximal activity in L-15B (Fig. 2). This threshold is twice that determined previously for *D. punctata* CA in TC199 (Tobe and Clarke, 1985), and we suggest that this difference is due to the 60% higher activity of CA used in our study. The need for higher methionine concentrations by more highly active CA was first noted by Tobe and Clarke (1985), who found that CA with very low activity (8–10 pmol JH h^{-1}) required only 20 μ*M* L-met to attain maximal rates of JH biosynthesis, whereas glands of moderate activity (80 pmol JH h^{-1}) required at least 30 μ*M* L-met. On the basis of our current results, we have chosen to use 100 μ*M* L-met in radiochemical assays with *D. punctata* CA. Reevaluation of this concentration will be necessary if future modifications of culture conditions lead to further enhancement of CA activity.

Use of L-15B in short-term assays. Results from short-term *in vitro* assays have been used to infer *in vivo* rates of JH synthesis (Feyereisen, 1985). However, *in vitro* biosynthetic rates are largely influenced by the choice of incubation medium. For example, in different media, CA from *T. molitor* synthesize JH at rates varying by an order of magnitude (Weaver et al., 1980), and in *L. decemlineata*, CA produce three to four times more JH in MEM than in TC199 (Khan et al., 1982). These results indicate that it is imperative to identify an optimal medium before proceeding with studies that draw inferences about *in vivo* CA activity from *in vitro* assays.

Our selection of L-15B was based primarily upon its consistently successful application in insect cell and organ culture. Cell lines from such diverse arthropods as ticks, moths, mosquitoes, cockroaches, and grasshoppers have been grown in L-15B (Munderloh et al., 1994), and cells within CA of *D. punctata* have been shown to proliferate in L-15B containing fetal bovine serum (FBS) and cockroach hemolymph (Tsai et al., 1995). Moreover, biosynthetic activity of CA from *L. migratoria* is retained for up to 30 d in L-15B fortified with FBS and tryptose phosphate broth (Gadot et al., 1993). An additional motivation for our selection of L-15B was its much greater overall similarity to cockroach hemolymph than TC199. The osmotic pressures of L-15B (362 mOsm/l) and *D. punctata* hemolymph (360 mOsm/l) are nearly identical, whereas the osmotic pressure of TC199 is much lower at only 288 mOsm/l. Also, the concentration of sugars, amino acids, and organic acids in L-15B greatly exceeds that in TC199 and much more closely resembles that of cockroach hemolymph (Woodring, 1985).

In short-term incubations, both sequential (Fig. 3) and split-gland assays showed that L-15B promoted JH biosynthesis by about 13% at 27° C. The fact that glands in L-15B were better able to respond to elevation of incubation temperature to 30° C provided further evidence of the superior nature of L-15B. These results indicate that TC199 should be replaced with L-15B in short-term radiochemical assays with *D. punctata* CA. We have recently used L-15B to determine patterns of JH biosynthesis by CA from *D. punctata* embryos (Holbrook et al., 1996).

Use of L-15B in long-term culture. Our results clearly show that L-15B is more appropriate than TC199 for long-term CA culture. During a 48-h incubation, JH biosynthesis decreased at a much faster rate in TC199 than in L-15B, and cumulative JH production in L-15B was 55% higher than in TC199 (Fig. 5). Rapid decline in CA activity in TC199 had been noted previously in other cockroach species. For example, in *B. germanica* the rate of JH synthesis in TC199 was reported to fall to one-third of its initial value between 12 and 24 h (Bellés et al., 1987). CA from *D. punctata* have been reported to release JH at constant rates for up to 24 h in TC199 (Stay and Tobe, 1977; Tobe et al., 1981), but in these studies only CA with low activity (15–25 pmol JH h^{-1}) were examined and after 24 h JH release by even these glands declined (Stay and Tobe, 1977). Pratt et al. (1976) also found that low activity CA from *P. americana* maintained linear JH release rates for long intervals in TC199, but highly active CA lost half of their initial activity within 24 h.

Because the compositions of L-15B and TC199 differ greatly, several factors could have accounted for the disparity in CA activity between the media. We hypothesized, however, that the high concentration of sugars, amino acids, and organic acids in L-15B was largely responsible for better maintenance of gland activity in this medium. Our speculation was based upon the absolute dependence

of JH biosynthesis on the presence carbon-containing substrates in the medium (Feyereisen and Farnsworth, 1987).

When TC199 was supplemented with different components found in L-15B, retention of CA activity in a 36-h incubation was only slightly enhanced by addition of 6.9 m*M* glucose and 5 m*M* galactose or 5 m*M* pyruvic acid and 2 m*M* α-ketoglutaric acid but was significantly improved by addition of 34 m*M* various amino acids (Table 1). Two factors could have accounted for this difference. First, amino acids may have been more effectively used for JH biosynthesis than sugars and organic acids. Second, CA activity may have been higher in TC199 with amino acids because a higher molar quantity of amino acids than sugars and organic acids was added to the medium. To address the second hypothesis, we supplemented TC199 with 34 m*M* glucose but were surprised to discover that JH biosynthesis in this formulation was depressed to a lower level than in unmodified TC199 containing only 5.5 m*M* glucose. Because glucose is normally found only at micromolar concentrations in cockroach hemolymph (Matthews et al., 1976), we cultured CA in TC199 containing 34 m*M* trehalose, the predominant hemolymph sugar in cockroaches (Woodring, 1985; Feyereisen and Farnsworth, 1987). With trehalose, CA retained similar levels of activity as glands in TC199 with 34 m*M* amino acids. This indicates that amino acids are not qualitatively unique in their ability to support long-term CA activity. Qualitative differences do exist, however, between sugars because the same concentrations of glucose and trehalose supported different levels of JH biosynthesis.

Suppression of CA activity by 34 m*M* glucose was unexpected because L-15B with 80 m*M* glucose is an excellent medium for culture of cockroach cells (Munderloh et al., 1994). Our results suggest that function of cockroach tissues, if not proliferation of cockroach cells, is impaired by high glucose concentrations. This hypothesis is supported in part by Kunkel et al. (1989), who found that nonphysiological glucose levels inhibited vitellogenin uptake by cockroach ovarioles. Because the glucose concentration in L-15B far exceeds that in cockroach hemolymph, we speculated that decreasing glucose levels in L-15B might improve CA function. Our results showed that retention of CA activity in a 36-h incubation was slightly but not significantly better in L-15B with 1 m*M* glucose and 34 m*M* trehalose than in standard L-15B containing 12.4 m*M* glucose (Table 2). Nevertheless, we believe that long-term CA culture is best performed in L-15B with reduced glucose and increased trehalose, because this formulation more closely resembles cockroach hemolymph.

Proper osmotic conditions are often critical for retention of cell function *in vitro*. For example, Kurtti et al. (1974) found that growth of *Heliothis* (≡ *Helicoverpa*) *zea* cells was optimal in media isoosmotic with *H. zea* hemolymph but impaired at nonphysiological osmotic pressures. Because the osmotic pressure of TC199 (288 mOsm/l) was much lower than that of *D. punctata* hemolymph (360 mOsm/l), we speculated that elevating the osmolality of TC199 would improve CA function. When TC199 was adjusted to 366 mOsm/l with NaCl, the rate of decline in CA activity was significantly lessened between 12 and 24 h of a 36-h incubation (Table 1). Although this result suggests that isoosmotic conditions enhance long-term CA activity, gland function may have been improved by elevation of sodium concentration. Nevertheless, Kurtti et al. (1975) showed that wide fluctuations in sodium levels had little effect on cell growth. Thus, osmotic effects of NaCl likely had much greater impact on CA function than elevation of sodium ion concentration. In any case, JH biosynthetic rates in TC199 at 366 mOsm/l collapsed after 24 h to low levels similar to those by CA in unmodified TC199. Therefore, both proper osmotic conditions and abundant nutrients are important for retention of CA function *in vitro*.

Although CA in L-15B maintain high biosynthetic rates in long-term culture, gland activity in this medium nonetheless declines. Thus, further modifications of culture conditions should be pursued. Lipophorin, the JH-binding protein in cockroach hemolymph, may prove useful in CA culture. Lanzrein et al. (1993) found that this protein promoted linear release of JH from CA of *N. cinerea* in TC199. Lipids also require investigation. We have recently found that a lipid mixture substantially elevates CA activity in short-term incubations (unpublished results).

Establishment of a culture system in which *in situ* CA function can be retained for extended periods *in vitro* would benefit studies on regulation of CA activity through slow developmental changes in glandular biosynthetic machinery (Chiang et al., 1991). To date, only a medium containing 80% hemolymph has been shown capable of maintaining high levels of JH biosynthesis beyond 24 h (Wilhelm et al., 1987). Because such a medium contains numerous undefined components, it is unacceptable for studies on CA regulation. L-15B with trehalose is capable of sustaining high rates of activity by most *D. punctata* CA. In this medium, CA from females with oocyte lengths of 1.15–1.24 mm maintained over 80% of initial activity during the third 12-h interval of a 36-h incubation (Table 2), and glands from females with smaller oocytes retained 100% of initial activity (unpublished results). JH biosynthetic rates appear difficult to sustain only in glands approaching the declining phase in CA activity (Table 1). These CA from females with oocyte lengths of 1.25–1.54 mm may be exhausted of internal reserves and highly dependent upon medium nutrients for retention of function. Future studies on improvement of L-15B should continue to focus on these glands.

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References

Bellés, X.; Casas, J.; Messeguer, A., et al. *In vitro* biosynthesis of JH III by the corpora allata of adult females of *Blattella germanica* (L.). Insect Biochem. 17:1007–1010; 1987.

Borovsky, D.; Carlson, D. A. *In vitro* assay for the biosynthesis and metabolism of juvenile hormone by exposed corpora allata of *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 29:318–324; 1992.

Chiang, A.-S.; Gadot, M.; Burns, E. L., et al. Developmental regulation of juvenile hormone synthesis: ovarian synchronization of volumetric changes of corpus allatum cells in cockroaches. Mol. Cell. Endocrinol. 75:141–147; 1991.

Chino, H.; Sakurai, S.; Ohtaki, T., et al. Biosynthesis of α-ecdysone by prothoracic glands *in vitro*. Science 183:529–530; 1974.

Cusson, M.; McNeil, J. N.; Tobe, S. S. *In vitro* biosynthesis of juvenile hormone by corpora allata of *Pseudoletia unipuncta* virgin females as a function of age, environmental conditions, calling behaviour and ovarian development. J. Insect Physiol. 36:139–146; 1990.

Feyereisen, R. Regulation of juvenile hormone titer: synthesis. In: Kerkut, G. A.; Gilbert, L. I., ed. Comprehensive insect physiology, biochemistry, and pharmacology. Vol. 7. Oxford, England: Pergamon Press; 1985:391–429.

Feyereisen, R.; Farnsworth, D. E. Precursor supply for insect juvenile hormone III biosynthesis in a cockroach. J. Biol. Chem. 262:2676–2681; 1987.

Feyereisen, R.; Friedel, T.; Tobe, S. S. Farnesoic acid stimulation of C16 juvenile hormone biosynthesis by corpora allata of adult female *Diploptera punctata*. Insect Biochem. 11:401–409; 1981.

Feyereisen, R.; Tobe, S. S. A rapid partition assay for routine analysis of juvenile hormone release by insect corpora allata. Analyt. Biochem. 111:372–375; 1981.

Gadot, M.; Applebaum, S. W. Rapid *in vitro* activation of corpora allata by extracted locust brain allatotropic factor. Arch. Insect Biochem. Physiol. 2:117–129; 1985.

Gadot, M.; Pener, M. P.; Schal, C. Variability in juvenile hormone production by locust corpora allata kept *in vitro* for long periods. Physiol. Entomol. 18:257–262; 1993.

Grace, T. D. C. Development of insect cell culture. In: Maramorosch, K.; Mituhashi, J., ed. Invertebrate cell culture applications. New York: Academic Press; 1982:1–8.

Granger, N. A.; Mitchell, L. J.; Niemiec, S. M. Biosynthesis of juvenile hormones I and III by the corpora allata of *Manduca sexta*: effect of *in vitro* conditions on gland activity. In: Techniques in the life sciences. Ireland: Elsevier Scientific Publishers; 1986: C212/1–C212/15.

Holbrook, G. L.; Chiang, A.-S.; Schal, C. Allatostatin inhibition and farnesol stimulation of corpus allatum activity in embryos of the viviparous cockroach, *Diploptera punctata*. Arch. Insect Biochem. Physiol. 32:341–352; 1996.

Khan, M. A.; Doderer, A.; Koopmanschap, A. B., et al. Improved assay conditions for measurement of corpus allatum activity *in vitro* in the adult Colorado potato beetle, *Leptinotarsa decemlineata*. J. Insect Physiol. 28:279–284; 1982.

Kikukawa, S.; Tobe, S. S.; Solowiej, S., et al. Calcium as a regulator of juvenile hormone biosynthesis and release in the cockroach *Diploptera punctata*. Insect Biochem. 17:179–187; 1987.

Kramer, S. J.; Law, J. H. Control of juvenile hormone production: the relationship between precursor supply and hormone synthesis in the tobacco hornworm, *Manduca sexta*. Insect Biochem. 10:569–575; 1980.

Kunkel, J. G.; Bowdan, E.; Kindle, H., et al. An inhibitory role of glucose in the vitellogenic process. In: Tonner, M.; Soldán, T.; Bennetton, B., ed. Regulation of insect reproduction IV. Praha: Academia Publishing House; 1989:85–86.

Kurtti, T. J.; Brooks, M. A. The dissociation of insect embryos for cell culture. In Vitro 12:141–146; 1976.

Kurtti, T. J.; Chaudhary, S. P. S.; Brooks, M. A. Influence of physical factors on the growth of insect cells *in vitro*. I. Effect of osmotic pressure on growth rate of a moth cell line. In Vitro 10:149–156; 1974.

Kurtti, T. J.; Chaudhary, S. P. S.; Brooks, M. A. Influence of physical factors on the growth of insect cells *in vitro*. II. Sodium and potassium as osmotic pressure regulators of moth cell growth. In Vitro 11:274–285; 1975.

Lanzrein, B.; Gentinetta, V.; Fehr, R., et al. Correlation between haemolymph juvenile hormone titre, corpus allatum volume, and corpus allatum *in vivo* and *in vitro* activity during oocyte maturation in a cockroach (*Nauphoeta cinerea*). Gen. Comp. Endocrinol. 36:339–345; 1978.

Lanzrein, B.; Wilhelm, R.; Riechsteiner, R. Differential degradation of racemic and 10R-juvenile hormone III by cockroach (*Nauphoeta cinerea*) haemolymph and the use of lipophorin for long-term culturing of corpora allata. J. Insect Physiol. 39:53–63; 1993.

Matthews, J. R.; Downer, R. G. H.; Morrison, P. E. Estimation of glucose in the haemolymph of the American cockroach, *Periplaneta americana*. Comp. Biochem. Physiol. 53A:165–168; 1976.

Munderloh, U. G.; Kurtti, T. J. Formulation of medium for tick cell culture. Exp. Appl. Acarol. 7:219–229; 1989.

Munderloh, U. G.; Kurtti, T. J.; Liu, T., et al. Grasshopper cell culture. In: Maramorosch, K.; Mituhashi, J., ed. Arthropod cell culture systems. Boca Raton, Florida: CRC Press; 1994:51–64.

Pratt, G. E.; Tobe, S. S. Juvenile hormones radiobiosynthesized by corpora allata of adult female locusts *in vitro*. Life Sci. 14:575–586; 1974.

Pratt, G. E.; Tobe, S. S.; Weaver, R. J., et al. Spontaneous synthesis and release of C_{16} juvenile hormone by isolated corpora allata of female locust *Schistocerca gregaria* and female cockroach *Periplaneta americana*. Gen. Comp. Endocrinol. 26:478–484; 1975.

Pratt, G. E.; Weaver, R. J.; Hamnett, A. F. Continuous monitoring of juvenile hormone release by superfused corpora allata of *Periplaneta americana*. In: Gilbert, L. I., ed. The juvenile hormones. New York: Plenum Press; 1976:164–178.

Richard, D. S.; Applebaum, S. W.; Sliter, T. J., et al. Juvenile hormone bisepoxide biosynthesis *in vitro* by the ring gland of *Drosophila melanogaster*: a putative juvenile hormone in the higher Diptera. Proc. Natl. Acad. Sci. USA 86:1421–1425; 1989.

Schal, C.; Chiang, A.-S.; Burns, E. L., et al. Role of the brain in juvenile hormone synthesis and oocyte development: effects of dietary protein in the cockroach *Blattella germanica* (L.). J. Insect Physiol. 39:303–313; 1993.

Schooley, D. A.; Baker, F. C. Juvenile hormone biosynthesis. In: Kerkut, G. A.; Gilbert, L. I., ed. Comprehensive insect physiology, biochemistry, and pharmacology. Vol. 7. Oxford, England: Pergamon Press; 1985:363–389.

Stay, B.; Tobe, S. S. Control of juvenile hormone biosynthesis during the reproductive cycle of a viviparous cockroach I. Activation and inhibition of corpora allata. Gen. Comp. Endocrinol. 33:531–540; 1977.

Stay, B.; Tobe, S. S.; Bendena, W. G. Allatostatins: identification, primary structures, functions and distribution. Adv. Insect. Physiol. 25:267–338; 1994.

Tobe, S. S.; Clarke, N. The effect of L-methionine concentration on juvenile hormone biosynthesis by corpora allata of the cockroach *Diploptera punctata*. Insect Biochem. 15:175–179; 1985.

Tobe, S. S.; Pratt, G. E. The influence of substrate concentrations on the rate on insect juvenile hormone biosynthesis by corpora allata of the desert locust *in vitro*. Biochem. J. 144:107–113; 1974.

Tobe, S. S.; Stay, B. Structure and regulation of the corpus allatum. Adv. Insect Physiol. 18:305–432; 1985.

Tobe, S. S.; Stay, B.; Friedel, T., et al. The role of the brain in regulation of the corpora allata in female *Diploptera punctata*. In: Pratt, G. E.; Brooks, G. T., ed. Juvenile hormone biochemistry. New York: Elsevier/North Holland Biomedical Press; 1981:161–174.

Tsai, W.-H.; Holbrook, G. L.; Schal, C., et al. *In vitro* growth of corpora allata from *Diploptera punctata*. In Vitro Cell. Dev. Biol. 31A:542–547; 1995

Weaver, R. J.; Pratt, G. E.; Hamnett, A. F., et al. The influence of incubation conditions on the rates of juvenile hormone biosynthesis by corpora allata isolated from adult females of the beetle *Tenebrio molitor*. Insect Biochem. 10:245–254; 1980.

Wilhelm, R.; Riechsteiner, R.; Lanzrein, B. On the competence of corpora allata to synthesise juvenile hormone and the dependence of their activity on haemolymph factors in the cockroach, *Nauphoeta cinerea*. J. Insect Physiol. 17:971–975; 1987.

Woodring, J. P. Circulatory systems. In: Blum, M., ed. Fundamentals of insect physiology. New York: John Wiley & Sons; 1985:5–57.

Zou, B.-X.; Yin, C.-M.; Stoffolano, J. G., et al. Juvenile hormone biosynthesis and release during oocyte development in *Phormia regina* Meigen. Physiol. Entomol. 14:233–239; 1989.