MAINTENANCE OF IMAGINAL DISCS OF *DROSOPHILA MELANOGASTER* IN CHEMICALLY DEFINED MEDIA

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ABSTRACT

A phosphate-buffered saline and a chemically defined synthetic medium for in vitro maintenance of imaginal discs of *Drosophila melanogaster* were developed. The composition of the chemically defined medium was varied in order to optimize the incorporation of tritiated uridine into RNA and tritiated amino acids into acid-insoluble protein. The optimal ranges obtained were: pH, 6.75–7.35; osmolarity, 285–345 milliosmoles/liter; sodium concentration, 40–60 mM/liter; potassium concentration, 40–60 mM/liter; magnesium concentration, 0.5–3.5 mM/liter; calcium concentration, 0.3–1.5 mM/liter; and inorganic phosphate concentration, 1.5–4.0 mM/liter. The phosphate-buffered saline is superior to a commonly used insect Ringer solution in maintaining total RNA and acid-insoluble protein synthesis in culture. The chemically defined synthetic medium permits linear total RNA and acid-insoluble protein synthesis for more than 48 hr, DNA synthesis for several hours, normal differentiation to occur after 74 hr in vitro, and trypsinization of imaginal discs into single cell suspensions without developmental damage.

The amount of genetic information about *Drosophila melanogaster* is extensive and many mutant strains are available for research. If appropriate tissue culture systems can be developed for *Drosophila*, like those already well established in vertebrate systems, numerous problems in cell physiology, developmental biology, neurobiology, and comparative biochemistry could be explored to an extent not presently possible.

Recent reviews (1–3) have stressed the difficulty of in vitro cultivation of insect organs, tissues, and cells. Some success has been achieved in utilizing embryonic cells of *Drosophila* (4–10) and imaginal discs of *Drosophila* (11–13). Media used in these systems have the serious drawback of not being completely defined; they contain sera, extracts, or hydrolysates of unknown composition. Embryonic cell systems have a further possible complication in that cells used in these cultures are a mixture of embryonic cell types whose individual developmental destinies have not been determined (4).[1] Use of the larval imaginal disc, which is the morphologically recognizable anlage of a given adult structure (e.g. wing, eye, leg), provides cells that are developmentally determined, are relatively homogeneous in their morphologic and developmental capacities, and can be cultured in vivo for many years without losing these characteristics (14–15).

The media used in the above systems were based to different extents on the published composition of the larval hemolymph (16–18). To the best of

[1] J. Sang Personal communication.

my knowledge, there are no quantitative studies on the composition of a chemically defined medium that will maintain the imaginal disc (or any other tissue of *Drosophila*) in a demonstrably normal, metabolically active state. Therefore, it was not possible to know whether the published hemolymph values were the best guidelines in the development of a suitable synthetic medium for *Drosophila*. Using a chemically defined synthetic medium, I have explored the ranges of pH, osmolarity, and concentrations of sodium, potassium, magnesium, calcium, and inorganic phosphate to find the optimal in vitro conditions for incorporation of tritiated uridine into total RNA and tritiated amino acids into acid-insoluble protein. Using these ranges, I have developed a phosphate-buffered saline and a chemically defined synthetic medium.

MATERIALS AND METHODS

Eggs of *D. melanogaster* containing young embryos homozygous for the markers ebony (*e*) and multiple wing hairs (*mwh*) were collected. Chorions were removed and the eggs' surfaces were sterilized by placing eggs for 2 min in 2.6% sodium hypochlorite (50% Clorox), then in 70% ethanol for 15 min, and finally rinsing them once with sterile distilled water. The eggs were then placed in sterile ¼ pint milk bottles prepared as follows: about 50 ml of standard cornmeal-molasses-agar-yeast extract medium with 0.12% methyl-p-hydroxybenzoate was placed in the bottle. Two ml of thick baker's yeast suspension was placed on top of the medium after solidification and the bottles were autoclaved. The embryos were allowed to develop at 25°C. The larvae were collected aseptically when they reached the climbing stage just before spiracle eversion and puparium formation. They were washed once with either sterile insect Ringer's (IR) (19) or *Drosophila* phosphate-buffered saline (DPBS) (Table I), followed by two washes with 70% ethanol and a final rinse with either sterile IR or DPBS. Insect Ringer's was used until DPBS was found to be superior, as discussed in the Results section. Wing imaginal discs of equal developmental maturity were dissected from larvae in either sterile IR or DPBS and transferred immediately with a sterile, fat-body–lubricated micropipette to an appropriate medium. Discs with injured membranes were discarded because membrane injury decreases the amount of uridine and amino acid incorporation.

The formulation of R-14 medium is given in Table II. The use of folic acid, putrescine dihydrochloride, vitamin B_{12}, lipoic acid, thymidine, $CuSO_4$, $ZnSO_4$, $FeSO_4$, and linoleic acid was suggested by their necessity in the first defined mammalian medium which would support clonal growth of cells (20). The use of the organic acids was suggested by the work of Wyatt (21) who demonstrated the importance of one or more of them in an early insect medium. All stock solutions were prepared with glass-distilled water, except solution 14-17 which was prepared in absolute ethanol. A 0.45 μ Millipore filter was used to sterilize all stock solutions except 14-5, 14-6, 14-7, 14-8, 14-10, and 14-11 which were autoclaved. All stock solutions were stored at 4°C except 14-2, 14-3, 14-12, 14-13, 14-14, 14-16, and 14-17 which were stored at −20°C. The medium was stirred at all times with a Teflon-coated magnetic stirring bar during its preparation. Initially, cholesterol was added dry to the mixing container. Next, stock solutions were added in the order listed, with special care that solutions 14-7, 14-8, and 14-9 were added very slowly in order to prevent precipitation of magnesium and/or calcium phosphate and/or hydroxide. After all stock solutions were mixed, except solution 14-17, the medium was filtered simultaneously through a Millipore prefilter and a 1.2 μ Millipore filter to remove the undissolved cholesterol. Glass-distilled water was then added to make up the desired volume (e.g., 1000 ml). Sterilization was accomplished by filtration through a 0.45 μ Millipore filter. The medium was stored at −20°C. Solution 14-17 was added just before use. Phenol red and antibiotics may be omitted if desired.

Culture vessels were disposable plastic multi-well

TABLE I

Composition of Drosophila Phosphate-Buffered Saline

Stock solution	Compound	Concentration in DPBS
		moles/liter
A* (2 ×)	NaCl	5.2×10^{-2}
	KCl	4.0×10^{-2}
	Glucose	1.0×10^{-2}
	Sucrose	1.0×10^{-1}
	$MgSO_4 \cdot 7H_2O$	1.2×10^{-3}
	$MgCl_2 \cdot 6H_2O$	1.2×10^{-3}
	$CaCl_2 \cdot 2H_2O$	1.0×10^{-3}
B* (2 ×)	Na_2HPO_4	2.0×10^{-3}
	KH_2PO_4	3.7×10^{-4}
	NaOH‡	2.0×10^{-4}

* Only glass-distilled water was used in the stock solutions. Stock solutions were autoclaved separately and mixed in equal volumes (1:1) just before use. Stock solutions were stored at room temperature.

‡ NaOH can be added to solution B as 0.10 N NaOH in glass-distilled water, 4.0 ml/liter of solution B. The pH can be varied by varying the amount of NaOH added to solution B.

trays (Linbro Chemical Co. New Haven, Conn.) sterilized with ultraviolet irradiation. Each well contained 0.1 ml of medium and two wing discs. Identical cultures were made by taking four discs from two larvae and placing one disc from each larva into each of two wells. One of the identical wells was used for uridine incorporation and the other for amino acid incorporation. All cultures were maintained at 27°C.

TABLE II

Composition of R-14 Medium

Stock solution	Compound	Concentration in R-14
		moles/liter
No stock*	Cholesterol	Saturated at 23°C
14-1 (10 ×)	L-Alanine	1.6×10^{-4}
	L-Asparagine	2.0×10^{-4}
	L-Aspartic acid	1.6×10^{-4}
	L-Proline	3.2×10^{-4}
	L-Serine	1.6×10^{-4}
	Glycine	1.6×10^{-4}
	β-Alanine	1.0×10^{-4}
14-2 (10 ×)	L-Arginine HCl	2.0×10^{-3}
	L-Glutamine	4.0×10^{-3}
	L-Histidine HCl	4.0×10^{-4}
	L-Isoleucine	6.4×10^{-4}
	L-Leucine	6.4×10^{-4}
	L-Lysine HCl	8.0×10^{-4}
	L-Methionine	2.0×10^{-4}
	L-Phenylalanine	4.0×10^{-4}
	L-Threonine	7.4×10^{-4}
	L-Tryptophan	8.0×10^{-5}
	L-Tyrosine	3.2×10^{-4}
	L-Valine	6.4×10^{-4}
14-3 (10 ×)‡	L-Cystine HCl	2.6×10^{-4}
14-4 (10 ×)	Fumaric acid (Na salt)	1.5×10^{-3}
	α-Ketoglutaric acid (Na salt)	1.9×10^{-3}
	Malic acid	4.3×10^{-3}
	Pyruvic acid (Na salt)	8.0×10^{-4}
	Succinic acid (Na salt)	4.5×10^{-3}
14-5 (0.25 N)§	NaOH	Approx. 2.5×10^{-2}
14-6 (10 ×)	Glucose	1.0×10^{-2}
	Sucrose	8.8×10^{-2}
14-7 (10 ×)	$MgSO_4 \cdot 7H_2O$	1.2×10^{-3}
	$MgCl_2 \cdot 6H_2O$	1.2×10^{-3}
14-8 (10 ×)	$CaCl_2 \cdot 2H_2O$	1.2×10^{-3}
14-9 (10 ×)	NaCl	2.5×10^{-2}
	KCl	3.8×10^{-2}
	Na_2HPO_4	2.0×10^{-3}
	KH_2PO_4	3.7×10^{-4}
14-10 (100 ×)	$FeSO_4 \cdot 7H_2O$	2.4×10^{-6}
14-11 (1000 ×)	$ZnSO_4 \cdot 7H_2O$	2.4×10^{-6}
14-12 (100 ×)	Lipoic acid	8.0×10^{-8}
	Thymidine	2.4×10^{-6}
	$CuSO_4 \cdot 5H_2O$	8.0×10^{-8}
14-13 (100 ×)	Biotin	2.5×10^{-8}
	Choline chloride	1.0×10^{-4}
	Folic acid	2.0×10^{-6}
	myo(i)-Inositol	7.8×10^{-4}
	Nicotinamide	6.6×10^{-6}
	D-Ca Pantothenate	3.3×10^{-6}
	Para-aminobenzoic acid	6.0×10^{-6}
	Pyridoxal HCl	4.6×10^{-6}
	Riboflavin	2.0×10^{-7}
	Thiamine HCl	2.6×10^{-6}
14-14 (100 ×)	Vitamin B_{12}	8.0×10^{-7}
	Putrescine dihydrochloride	8.0×10^{-7}
14-15 (1000 ×)	Phenol red	3.3×10^{-6}
14-16 (100 ×)	Penicillin	100 Units/ml
	Streptomycin	100 μg/ml
14-17 (1000 ×)‖	Linoleic acid	2.4×10^{-7}

* About 2 mg of dry cholesterol were added to mixing vessel.
‡ Discarded if precipitate formed upon thawing.
§ pH adjusted to 7.1 with 0.25 N NaOH.
‖ Prepared fresh each month by using absolute ethanol and added just before use.

The following radioactive precursors were used in the experiments exploring the ranges of pH, osmolarity, and concentrations of sodium, potassium, magnesium, calcium, and inorganic phosphate, and the experiments studying incorporation in DPBS and insect Ringer's. Uridine-5-^{3}H (Schwarz BioResearch Orangeburg, N. Y., specific activity: 20 c/mmole) was used at a concentration of 10 μc/ml to measure total RNA synthesis. A protein hydrolysate-^{3}H (Schwarz BioResearch) containing 16 amino acids-^{3}H with a total specific activity of 18 c/mmole was used at a concentration of 20 μc/ml for the measurement of acid-insoluble protein synthesis. The following radioactive precursors were used in the experiment exploring the incorporation of uridine, serine, and thymidine in R-14 medium as a function of time. Uridine-5-^{3}H (Schwarz BioResearch, specific activity: 20 c/mmole) was used at a concentration of 1.0 μc/ml to measure total RNA synthesis. L-Serine-^{3}H (Schwarz BioResearch, specific activity: 0.3 c/mmole) was used at a concentration of 1.0 μc/ml to measure acid-insoluble protein synthesis in R-14 medium containing only one-fifth the normal amount of L-serine (final specific activity: 0.06 c/mmole). Thymine-

methyl-^{3}H (Schwarz BioResearch, specific activity: 11 c/mmole) was used at a concentration of 1.0 μc/ml in R-14 medium without thymidine to measure DNA synthesis.

Before labeling, sterility of the disc cultures was checked by phase-contrast microscopy and by streaking duplicate cultures on nutrient agar medium and incubating at 37°C for 48 hr. Discs were transferred from unlabeled media to labeled media by a sterile, fat-body–lubricated micropipette. The incorporation was stopped after 2.5 hr at 27°C by adding 0.4 ml of cold 10% trichloroacetic acid directly to the incubation well. With a fat-body–lubricated micropipette and a dissecting microscope, discs were transferred to glass-fiber filters, both discs from one well onto one filter. Filters were individually washed with 10 ml of cold 5% trichloroacetic acid, 10 ml of 95% ethanol and 5 ml of acetone, and allowed to air dry. Next, they were incubated in vials with 0.1 ml of 25% Hyamine in absolute methanol at 60°C for 10 min. After cooling, 5 ml of scintillation fluid (toluene containing 0.5 gm/liter *p*-bis[2-(5-phenyloxazolyl)]benzene [POPOP] and 1.5 gm/liter polyphenylene oxide [PPO]) were added and the contents of the vials were counted in a Nuclear-Chicago Mark I Scintillation System. As a control for nonincorporated tritium, an equivalent amount of the labeled medium (2λ) was placed on a glass-fiber filter and washed in the same manner and its count was subtracted from the disc value. All counts were converted to disintegrations per minute in order to compare experiments.

RESULTS

The ranges of pH, osmolarity, and concentrations of sodium, potassium, magnesium, calcium, and inorganic phosphate that were examined for their effect on total RNA and acid-insoluble protein synthesis are given in Table III. The in vivo incorporation of the tritiated precursors into their respective macromolecules has not been examined for the wing imaginal disc of *D. melanogaster*. Therefore, a reference point (zero time) was established for each experiment by placing the dissected discs directly into the appropriate labeled insect Ringer's. Though these values may not reflect the true in vivo incorporation, they do represent a reasonable base line. The duplicate cultures were exposed to the tritiated precursors for the same period of time, after a 24 hr incubation in unlabeled R-14 medium.

Table III shows the percentage of the zero-time incorporation that remained after 24 hr in R-14 medium. Only the parameter under investigation was varied in the medium. The maintenance of uridine and amino acid incorporation was relatively uniform in a pH range between 6.75 and 7.35. A previous measure of the pH of third instar larval hemolymph is 6.6–6.7 (16).

Osmolarity was calculated by multiplying the molar concentration of a compound by the number of particles per mole obtained by ionization of that compound, assuming that complete ionization occurred. This assumption is reasonably accurate for the ionizable compounds used in R-14 medium. The optimal range of osmolarity for uridine incorporation was between 285 and 345 milliosmoles/liter, while that for amino acid incorporation was relatively uniform between 285 and 365 milliosmoles/liter. There was a decrease in the amount of both incorporations as the osmolarity was increased above 365 milliosmoles/liter. The measured value for osmolarity of third instar larval hemolymph is 360 milliosmoles/liter (16).

When sodium and potassium concentrations were varied as given in Table III, uridine incorporation increased as the potassium concentration increased and the sodium concentration decreased. Amino acid incorporation, however, had an optimal range centering around the measured third instar larval hemolymph values of 40 mM/liter potassium and 56 mM/liter sodium (16–17).

Magnesium and calcium concentrations were varied together with a ratio of magnesium to calcium of about 2.5:1.0 similar to the measured ratio in the third instar larval hemolymph (16). The ratio was also reversed (0.6:1.0), similar to that of mammalian media. As the magnesium and calcium concentrations increased, an optimal concentration for uridine incorporation was reached around 4–5 mM/liter magnesium and 1.5–2.0 mM/liter calcium. A rather striking inhibition of uridine incorporation occurred at the highest level tested (13.2 mM/liter magnesium, 5.1 mM/liter calcium), which is only about 0.6 times the estimated third instar larval hemolymph values of 21 mM/liter magnesium and 8 mM/liter calcium (16). Amino acid incorporation, however, started to decrease at concentrations that were optimal for uridine incorporation and was markedly inhibited at the highest level tested. When the magnesium to calcium ratio was reversed similar to that of mammalian media (0.6 mM/liter magnesium, 1.0 mM/liter calcium), both uridine and amino acid incorporations were lower than comparable incorporations in the

TABLE III

Uridine-^3H and Amino Acid-^3H Incorporation in Wing Imaginal Discs of D. melanogaster after 24 Hr of Incubation at 27°C in R-14 Medium with Varied Composition

Parameter	Zero-time incorporation (ZT)		24 hr incorporation (ZT ± SD)	
	Uridine-^3H	Amino acid-^3H	Uridine-^3H	Amino acid-^3H
pH	*dpm* ± SD	*dpm* ± SD	%	%
6.75*	2100 ± 21	3350 ± 540	100 ± 13	29 ± 3
6.95	(= 100%)	(= 100%)	87 ± 7	26 ± 3
7.20			83 ± 7	26 ± 2
7.35			82 ± 1	23 ± 3
Osmolarity mosm				
285	4000 ± 160	2700 ± 485	100 ± 6	45 ± 2
315			100 ± 6	30 ± 8
345			88 ± 6	36 ± 1
365*			38 ± 7	36 ± 2
400			16 ± 1	19 ± 1
K^+ *mM/liter* Na^+ *mM/liter*				
25 140	3930 ± 200	3210 ± 490	39 ± 1	24 ± 8
25 100			36 ± 1	37 ± 8
40* 56			67 ± 11	44 ± 8
50 50			76 ± 16	36 ± 7
80 56			99 ± 4	22 ± 1
100 25			88 ± 8	28 ± 1
Mg^{++} *mM/liter* Ca^{++} *mM/liter*				
0.6 0.3	3960 ± 200	2850 ± 315	72 ± 3	30 ± 4
1.4 0.6			67 ± 2	23 ± 2
2.7 1.1			78 ± 6	24 ± 4
3.5 1.4			98 ± 20	24 ± 3
4.8 1.9			100 ± 2	15 ± 1
13.2‡ 5.1			25 ± 5	3 ± 1
0.6 1.0	4000 ± 160	2700 ± 485	85 ± 5	30 ± 8
2.5 1.0			100 ± 2	40 ± 1
PO_4 *mM/liter*				
1.8	3850 ± 270	2750 ± 550	66 ± 8	36 ± 6
3.6*			72 ± 4	43 ± 1
7.2			92 ± 15	22 ± 2

For the medium in each experiment, only the parameter under study was varied (e.g., only the pH was varied in the pH experiment): pH was varied with 0.1 N NaOH or 0.1 N HCl; osmolarity with sucrose; sodium concentration with NaCl; potassium concentration with KCl; magnesium concentration with $MgCl_2$; calcium concentration with $CaCl_2$; and inorganic phosphate concentration with Na_2HPO_4 and KH_2PO_4, Each value in the table is given with its standard deviation (SD). The incorporated radioactivity is given in disintegrations per minute (*dpm*).

* Published values for third instar larval hemolymph (16–17).

‡ 0.6 times the published values of magnesium and calcium for third instar larval hemolymph (16).

TABLE IV

Ability of the Wing Imaginal Disc of D. melanogaster to Incorporate Uridine-^{3}H or Amino-Acid-^{3}H as a Function of Time in DPBS or IR Medium at 27°C

Length of incubation	Uridine-^{3}H incorp		Amino acid-^{3}H incorp.	
	in DPBS	in IR	in DPBS	in IR
hr	*dpm* ± SD	*dpm* ± SD	*dpm* ± SD	*dpm* ± SD
0	6 ± 2	3 ± 3	5 ± 3	4 ± 2
2.5	2,650 ± 16	3,030 ± 394	5,590 ± 567	7,320 ± 924
7.5	10,250 ± 55	4,850 ± 483	9,700 ± 296	10,400 ± 718
23.0	7,540 ± 980	1,690 ± 90	10,250 ± 72	6,190 ± 711

The values are given in disintegrations per minute (*dpm*) along with their standard deviations (SD).

medium with the ratio similar to that of hemolymph (2.5 mM/liter magnesium, 1.0 mM/liter calcium). This difference may not be significant since it was only tested once in triplicate.

When inorganic phosphate was varied as indicated in Table III, there was probably no significant change in uridine incorporation. However, amino acid incorporation was optimal at concentrations of 1.8–3.6 mM/liter. The latter concentration is close to the measured third instar larval hemolymph value of 3 mM/liter (16).

On the basis of the above culture conditions, a phosphate-buffered saline, DPBS (Table I), was developed. Total RNA and acid-insoluble protein synthesis were compared in DPBS and the IR of Ephrussi and Beadle (19). Duplicate cultures of wing imaginal discs were incubated in both DPBS and IR for 2.5, 7.5, and 23 hr at 27°C. Both salines contained identical amounts of uridine-^{3}H or amino acid-^{3}H. The IR contained 0.037 moles/liter of sucrose, which increased its osmolarity to that of DPBS and 0.004 moles/liter of glucose that was used as an energy source similar to that of DPBS. Table IV shows that after 2.5 hr of incubation the uridine incorporation in both salines was similar. After 7.5 hr the incorporation in DPBS had increased almost four times above its 2.5 hr value. In IR, however, the 7.5 hr incorporation was only 1.6 times greater than the 2.5 hr value. At 23 hr the incorporation in both salines had decreased, but the incorporation in DPBS was still 2.8 times the 2.5 hr value. Amino acid incorporation was similar in both salines at 2.5 and 7.5 hr. At 23 hr the incorporation in DPBS was equal to the 7.5 hr value and 1.8 times its 2.5 hr value. In contrast, the incorporation in IR was only 0.6 times the 7.5 hr value and 0.8 times the 2.5 hr value.

To test whether the low 23 hr incorporation values were due to increased degradation or decreased synthesis of RNA, triplicate cultures were incubated at 27°C in each of the two salines without any uridine-^{3}H. At 0, 2.5, 7.0, and 24 hr, the discs were transferred to uridine-^{3}H-labeled DPBS and IR identical with that used in the above experiment. Incorporation was stopped after 2 hr of incubation at 27°C. Only uridine-^{3}H was used because its incorporation was more sensitive to the length of time in culture than amino acid incorporation. The results are given in Table V. At 24 hr there was no detectable uridine incorporation and, therefore, no significant RNA synthesis in either DPBS or IR. Although the amount of incorporation was markedly decreased after only 2 hr of incubation (DPBS, 10% of the incorporation of discs immediately placed in the labeled DPBS after dissection; IR, 2.7%), there was still a fourfold difference between DPBS and IR.

These two experiments seem to demonstrate that DPBS was superior to IR in maintaining total RNA and acid-insoluble protein synthesis and in preventing the loss of this labeled RNA and protein from the wing imaginal disc cells. DPBS would thus be more suitable than IR for experiments in which imaginal discs or disc cells alone have to be exposed to in vitro conditions for a short time.

An experiment was performed to study the synthesis of total RNA, acid-insoluble protein, and DNA as a function of time in R-14 medium. Wing imaginal discs were cultured at 27°C in triplicate for each time point and for each tritiated precursor. The labeling was continuous for the time period involved. Figure 1 shows that uridine-^{3}H incorporation increased linearly for about 50 hr and

TABLE V

Ability of the Wing Imaginal Disc of D. melanogaster to Incorporate Uridine-^{3}H after Varying Preincubation Time in DPBS or IR Medium at 27°C

Preincubation time	Uridine-^{3}H incorporation	
	in DPBS	in IR
hr	*dpm* ± SD	*dpm* ± SD
0	5,370 ± 822	5,070 ± 795
2.5	546 ± 79	137 ± 27
7.0	460 ± 29	107 ± 41
24.0	3 ± 3	5 ± 3

The values are given in disintegrations per minute (*dpm*) along with their standard deviations (SD).

then began to plateau. Serine-^{3}H incorporation continued linearly for at least 44 hr. The low level of serine incorporation was possibly due to the low specific activity of the serine-^{3}H and to dilution by cellular serine. Thymidine-^{3}H incorporation continued for about 24 hr, after which the labeled DNA was slowly lost, presumably due to degradation. The radiation used (1.0 μc/ml) was a relatively high dose for such long incubation periods, so radiation damage may have been significant. The linearity of the RNA and protein synthesis is in agreement with the results of Fristrom et al. (12) except that those authors followed the incorporations for only 5 hr.

The most sensitive test for the metabolic and developmental normality of these discs after in vitro incubation is to implant them into third instar larvae and allow them to undergo metamorphosis (14, 19). The quality and quantity of normal adult structures formed from the preincubated, implanted discs as compared to discs implanted without preincubation, is a measure of their normality.

In experiments done in collaboration with W. Gehring, wing imaginal discs were incubated for 27, 50, and 75 hr in R-14 medium at 27°C and then implanted into larvae following the method of Ephrussi and Beadle (19). Other discs from the same group of larvae, but without preincubation, were similarly implanted and used as controls. After metamorphosis, the differentiated implants were removed from the adult hosts' abdomens and examined microscopically. All eight discs from the 27 hr incubation period and all eight discs from the 50 hr incubation period had adult wing disc structures identical with those of the controls in both quality and quantity. All five discs incubated

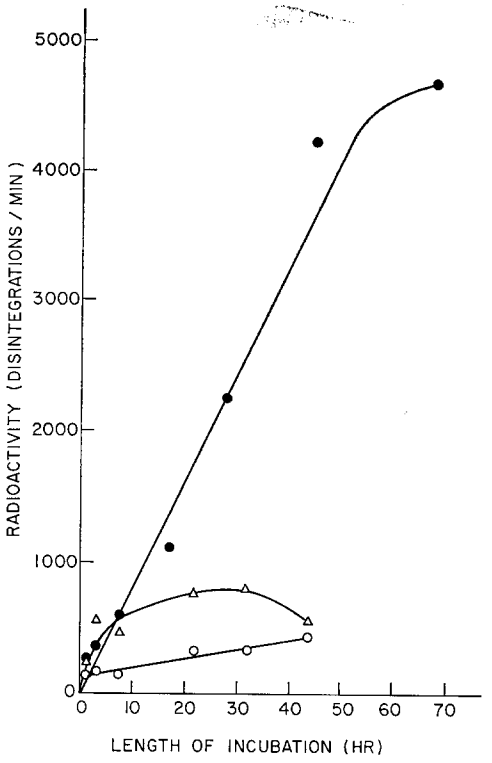

FIGURE 1 Ability of the wing imaginal disc of *D. melanogaster* to synthesize total RNA, acid-insoluble protein, and DNA as a function of time in R-14 medium at 27°C. Total RNA synthesis, as measured by the incorporation of uridine-^{3}H, is represented by the dots (●). The acid-insoluble protein synthesis, as measured by the incorporation of L-serine-^{3}H, is represented by the circles (○). The DNA synthesis, as measured by the incorporation of thymidine-^{3}H, is represented by the triangles (△).

for 74 hr had normally differentiated adult structures, but there was a 10–75% nonspecific loss of adult structures as compared to controls (e.g., wing components were not lost in preference to thoracic components). No abnormal structures were observed in any of the test implants. The loss of structures at 74 hr, without the appearance of any abnormal structures, represented either a loss of cells from the discs or a loss in the capacity of the cells to differentiate. After 74 hr in culture, an intact wing disc is difficult to implant because its surrounding membrane is partially broken down and many single cells and clumps of cells are lost during the implantation procedure. This loss of cells may account for the loss of adult structures after the 74 hr incubation. It is also possible that

some cells simply lost their capacity to differentiate or died because of the length of time in culture.

Although the wing discs are capable of normal differentiation after 74 hr in the R-14 medium, they show no observable differentiation while in the medium. However, the wing discs of 74-hr pupae have differentiated into essentially adult structures. Therefore, the developmental capacity of the discs is suspended by the R-14 medium. The experiments do not justify any statements as to what factor(s) might be responsible for this suspension, but it is interesting to speculate that the absence of ecdysone, the hormone responsible for metamorphosis, might play a significant role in this developmental suspension. This possibility could easily be tested.

With R-14 medium, imaginal discs from *D. melanogaster* have been trypsinized into single cell suspensions, reaggregated, and injected into larvae. The implanted cell aggregates metamorphosed to form normal adult structures appropriate to the imaginal disc used.[2]

DISCUSSION

Before tissue culture systems like those used for mammals can be developed for *D. melanogaster*, it is first necessary to see what culture conditions will permit tissues or cells of *Drosophila* to remain metabolically active. As a first approximation, the assumption was made that a cell which is capable of RNA and protein synthesis is metabolically active. Although this synthesis may or may not be normal metabolic activity for the cell in the larva, it can be measured and used to judge which in vitro conditions allow maximal synthesis and which conditions inhibit this synthesis.

The composition of larval and adult hemolymph is markedly dependent upon diet (e.g., in the adult there is no requirement for dietary calcium, but there is a strict requirement for dietary magnesium).[1] It is, therefore, necessary to find out if the published compositions of the third instar larval hemolymph (the environment in which the imaginal discs normally live and grow) provide reasonable guidelines for the development of media.

The capacity for RNA and protein synthesis by wing imaginal discs of *D. melanogaster* in culture was assayed by measuring the ability of the discs to incorporate uridine-^{3}H into total RNA and L-serine-^{3}H into acid-insoluble protein after 24 hr of incubation in a chemically defined synthetic medium, R-14. The pH, osmolarity, and concentrations of sodium, potassium, magnesium, calcium, and inorganic phosphate were varied independently in the medium. Ranges were found for these parameters which allowed optimal total RNA and acid-insoluble protein synthesis.

It is clear that wing imaginal disc cells do not synthesize RNA or protein efficiently in a medium that is constructed according to the published compositions of third instar larval hemolymph (16–18). Optimal synthesis occurred in a pH range of 6.75–7.35; an osmolarity range of 285–345 milliosmoles/liter; a magnesium concentration range of 0.5–3.5 mM/liter; and a calcium concentration range of 0.3 to 1.5 mM/liter. These values are closer to the values given for most mammalian culture media than to published values for hemolymph. The discs do, however, seem to synthesize RNA and protein more efficiently when the magnesium and calcium concentrations are in a ratio similar to that of third instar hemolymph (magnesium—2.5 mM/liter, calcium —1.0 mM/liter) than when the ratio is similar to that found in mammalian media (magnesium—0.6 mM/liter, calcium—1.0 mM/liter). The sodium concentration range of 40–60 mM/liter and the potassium concentration range of 40–60 mM/liter that allow optimal RNA and protein synthesis are similar to the values for hemolymph and are distinctly different from the values for mammalian media. The optimal inorganic phosphate concentration range of 1.5–4.0 mM/liter is similar to the values for both hemolymph and mammalian media.

The only sugars used in R-14 medium were glucose and sucrose. Although trehalose is the only sugar reported to be in third instar larval hemolymph (16), the disc cells did not require trehalose for at least 3 days. The amount of free amino acid in R-14 medium was about one-tenth the amount measured in third instar larval hemolymph (18). This suggests that imaginal disc cells may not need as much free amino acid in culture as the published values for hemolymph imply, but definitive experiments have not been done.

Although there are not enough experimental data to allow calculation of meaningful statistical significance, the trends are reasonably clear and provide quantitative guidelines for the develop-

[2] W. Gehring. Personal communication.

ment of better media in which the cells will continue to divide. The phosphate-buffered saline, DPBS, and the chemically defined synthetic medium, R-14, were developed by using the in vitro criteria described above as guidelines. DPBS maintained the wing imaginal disc cells in a more metabolically active state than did a commonly used insect Ringer's and should be useful for experiments that require short in vitro exposures. The R-14 medium should be useful in experiments that require longer incubations in a chemically defined medium. The R-14 medium permits linear total RNA and acid-insoluble protein synthesis for more than 48 hr, DNA synthesis for several hours, normal differentiation to occur after 74 hr in vitro, and trypsinization of imaginal discs into single cell suspensions without developmental damage.

These media and the assay used in these experiments can be used to study many facets of imaginal disc physiology, developmental mechanisms, and possible hormonal influence. It would be very useful to test the various components of the R-14 medium, one at a time, in order to develop a minimal medium. It would also be useful to find conditions and/or additives (e.g. sera, larval, or adult extracts) that would allow growth of imaginal disc cells in vitro.

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