EFFECTS OF SUBSTRATES ON GENE-CONTROLLED ENZYME ACTIVITIES IN CULTURED EMBRYONIC CELLS OF *DROSOPHILA*¹

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ONE of the central problems in the genetics of differentiation may be formulated in the following way: Why do embryonic cells possessing the necessary genetic information fail to synthesize certain specific proteins, and what are the mechanisms which are responsible for the initiation of such synthesis in particular cell lines at subsequent stages of development? The development of methods for *in vitro* culture of embryonic cells from *Drosophila melanogaster* has provided a tool for the study of this question (HORIKAWA and FOX 1964; HORIKAWA, LING, and FOX 1966). As a first step, the effects of substrates on the level of three different enzymes in cultured embryonic cells have been investigated.

The three enzymes chosen for this work were alkaline phosphatase, xanthine dehydrogenase, and alcohol dehydrogenase. Genetic and biochemical information is available for each of these enzymes, each is present at a very low level in the early embryo, and the level of each increases to reach a maximum at a specific stage in subsequent development. The question explored in the present work was whether the presence of substrate in culture medium could stimulate an increase in the level of any of these enzymes in embryonic cells. The results show that each enzyme responds in a different way to the presence of its substrate.

MATERIALS AND METHODS

Stocks: Three stocks of *D. melanogaster* were used in this work: (1) Oregon R-EL2—an inbred, wild-type stock used as a standard reference in this laboratory for many years. (2) rosy-2 (*ry*²)—a stock which is homozygous for a point mutant in the right part of the rosy cistron at 52.4 on the third chromosome. The eye color is deep ruby, and *ry*² flies exhibit no detectable xanthine dehydrogenase. (3) maroon-like (*ma-l*)—a stock which is homozygous for a mutant at 64± on the X chromosome. Eye color is dull ruby, and *ma-l* flies also lack detectable xanthine dehydrogenase. The stocks of *ry*² and *ma-l* were kindly provided by A. CHOVIČEK. Flies were grown at 22°C on sterile, standard cornmeal, molasses, agar medium containing killed brewer's yeast and propionate as a mold inhibitor, but unseeded with living yeast.

Cell culture methods: Suspensions of embryonic cells were prepared from eggs collected after a 6 hour oviposition period at 22°C according to the methods of HORIKAWA and FOX (1964) and HORIKAWA, LING, and FOX (1966). The cells were cultured at 30°C in complete H-5 medium (i.e., H-5 supplemented with 10% newborn calf serum) without shaking, and in normal atmosphere. In various experiments enzyme substrates were added to the medium as described. Changes

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of medium were accomplished by sedimentation of the cells by low-speed centrifugation followed by resuspension by gentle pipetting (HORIKAWA *et al.* 1966).

Chemicals: Carrier free (P^{32}O_4)³⁻ was obtained from the Oak Ridge National Laboratory at a concentration of 29.0 mC per ml. Appropriate corrections were made for decay during the course of the experiments. Hypoxanthine and xanthine were purchased from the California Corporation for Biochemical Research, and uric acid from Sigma Chemical Company: these compounds were checked for purity by means of paper chromatography. Nicotinamide adenine dinucleotide (NAD) was obtained from Pabst Laboratories. Tris(hydroxymethyl)aminomethane (Tris), *p*-nitrophenol, *p*-nitrophenyl phosphate, disodium phenylphosphate, DL- β -glycerophosphate (disodium salt pentahydrate), and 2-amino-2-methyl-1-propanol were all purchased from Sigma Chemical Company. Crystalline bovine albumin was obtained from Pentex Incorporated, and newborn calf serum from the Colorado Serum Company.

P^{32} -labeling experiments: Approximately 1.3×10^8 embryonic cells, freshly isolated from eggs of Oregon R-EL2, were equally divided into four T-60 flasks, each containing a total of 10 μC of carrier free (P^{32}O_4)³⁻ in 10 ml of complete H-5 medium. The flasks were incubated at 30°C for 1, 3, 6, and 24 hours respectively. At the end of the incubation period, the cells were collected by low-speed centrifugation, washed three times with basal H-5 medium (lacking calf serum), and counted. The acid-soluble, phospholipid, RNA, and DNA fractions of the collected cells were then isolated by the modified Schmidt-Thannhauser method proposed by SCHNEIDER (1946). The radioactivity of each fraction was determined on Whatman 3MM filter-paper discs (BOLLUM 1959) in a Packard Liquid Scintillation Spectrometer, Model 3314, and the phosphorus content of each was estimated by the method of HORECKER *et al.* (1940).

Alkaline phosphatase: Total alkaline phosphatase activity was assayed by an adaptation of the method of LOWRY *et al.* (1954). The substrate, *p*-nitrophenyl phosphate, is split to free *p*-nitrophenol which in alkaline solution has strong absorption at a wavelength (410 $m\mu$) at which the substrate has little or no absorption. An enzyme unit is defined as that amount which produces 1 μmole *p*-nitrophenol per minute in the presence of excess *p*-nitrophenyl phosphate.

The enzyme activity of cultured cells was determined by cooling single cultures to 5°C, collecting the cells by centrifugation, washing twice in ice-cold buffer (1M 2-amino-2-methyl-1-propanol, pH 10.0), and homogenizing in 2.0 ml of ice-cold buffer. Clarified enzyme extract was prepared from the crude homogenate by centrifugation at $5,000 \times g$ for 15 minutes at 5°C. The assay mixture consisted of 0.1 ml of enzyme extract and 0.5 ml of substrate-buffer (containing $2 \times 10^{-3}\text{M}$ MgCl_2 and $8 \times 10^{-3}\text{M}$ disodium *p*-nitrophenyl phosphate). The mixture was incubated 30 minutes at 38°C, rapidly cooled to 5°C, and 1.5 ml of 0.25N NaOH was added. Precipitated protein was removed by centrifugation at $1,100 \times g$ for 5 minutes, and optical density readings were made at 410 $m\mu$ after 30 minutes. The readings were corrected for zero time values and the amount of *p*-nitrophenol in the mixture was determined from a standard curve. *p*-Nitrophenol production in the assay mixtures was linear during the 30 minute duration of incubation at 38°C, and the assay was linear with enzyme concentration. Replicate determinations were accurate within limits of $\pm 5 \times 10^{-4}$ enzyme units.

For the determination of enzyme levels during development eggs (collected from several hundred females after an oviposition period of 6 hours), third instar larvae (20 to 30), 2-day pupae (20 to 30), or 2-day adults (15 males and 15 females) were homogenized in 2.0 ml of ice-cold buffer and clarified enzyme extract was prepared by centrifugation. In these cases, assay mixtures consisted of 0.1 ml of enzyme extract and 1.0 ml of substrate-buffer, to which 3.0 ml of 0.25N NaOH was added after 30 minutes incubation at 38°C. The amount of *p*-nitrophenol produced was determined from a separate standard curve.

Separate tests demonstrated that alkaline phosphatase from all stages of development can utilize β -glycerophosphate and phenylphosphate, as well as *p*-nitrophenyl phosphate, as substrate.

Alcohol dehydrogenase: Total alcohol dehydrogenase activity was assayed by an adaptation of the method of RACKER (1950). In the presence of enzyme 1 mole of substrate, isopropanol, is oxidized while 1 mole of NAD is reduced to NADH_2 . The latter has a molar absorption at 340 $m\mu$ of 6.22×10^3 . An enzyme unit is defined as that amount which results in the production of 1 μmole of NADH_2 per minute in the presence of excess isopropanol.

The enzyme activity of cultured cells was determined by cooling single cultures to 5°C, collecting the cells by centrifugation, washing twice in ice-cold buffer (0.2M Tris, pH 8.5), and homogenizing in 4.5 ml of ice-cold buffer. Clarified enzyme extract was prepared by centrifugation at $5,000 \times g$ for 15 minutes at 5°C, followed by passage of the supernatant through a coarse sintered glass filter. Enzyme activity was assayed at 25°C in mixtures with a total volume of 1.3 ml containing 0.95 ml of extract, 0.115M isopropanol, 2.0×10^{-3} M NAD, and 15×10^{-2} M Tris (pH 8.5). Enzyme units were calculated from the initial rate of change of optical density at 340 m μ , equating a change in optical density of 0.001 per minute with 2.09×10^{-4} enzyme units. The assay was linear with enzyme concentration and was accurate within limits of $\pm 2 \times 10^{-4}$ units. For the determination of enzyme levels during development, enzyme extracts were prepared in the same way and were assayed in the same fashion as cellular extracts.

Xanthine dehydrogenase: Xanthine dehydrogenase was assayed by an adaptation of the method of PARZEN and FOX (1964). In the presence of enzyme, 1 mole of hypoxanthine is oxidized to xanthine while 1 mole of NAD is reduced to NADH₂. The xanthine is oxidized in turn to uric acid in a second NAD-linked reaction. An enzyme unit is defined as that amount which results in the reduction of 1 μ mole NAD to NADH₂ per minute in the presence of excess hypoxanthine.

All steps in the extraction of enzyme from cultured cells were carried out at temperatures below 5°C. Cells were collected from individual cultures by centrifugation, washed twice in buffer (0.1M Tris, pH 8.0, containing 5 mg crystalline bovine albumin per ml), and homogenized in 1 ml of buffer. Norite-A (60 mg) was added to the homogenate, which was then allowed to stand for 60 minutes with occasional stirring. Clarified enzyme extract was prepared by centrifugation at $5,000 \times g$ for 15 minutes, followed by passage of the supernatant through a coarse sintered glass filter. Enzyme activity was assayed at 25°C in mixtures with a total volume of 1.12 ml containing 0.16 ml of enzyme extract, 1×10^{-4} M hypoxanthine, 3.5×10^{-3} M NAD, and 0.1M Tris (pH 8.0). Enzyme units were calculated from the initial rate of change of optical density at 340 m μ , equating a change in optical density of 0.001 per minute with 1.80×10^{-4} enzyme units. The assay was linear with enzyme concentration, and was accurate within limits of $\pm 10^{-4}$ units.

For the determination of enzyme levels during development, enzyme extracts were prepared by the same procedure as that used for cells, except that the initial homogenate was prepared in 1.5 ml of buffer. The extracts were assayed as described above.

Other methods: Initial reaction rates and all spectrophotometry was performed with a Beckman DU spectrophotometer equipped with a Gilford automatic sample changer and recorder.

Protein was determined by the method of LOWRY *et al.* (1951).

All experiments were repeated at least three times. The results of typical individual experiments are given here. In some cases the replicate experiments exhibited different absolute levels of enzyme activities, but the changes in activity reported here were consistently repeatable. The text specifies which of these changes are significant in magnitude, taking into consideration the sensitivity of the assay in each case.

RESULTS

RNA and DNA synthesis in cultured cells: The incorporation of P³² into phospholipid, acid-soluble, and nucleic acid fractions of embryonic cells in primary cultures was studied as a measure of their synthetic activities. For this purpose, cells were grown in T-60 flasks containing 10 ml of medium. Cultures were started with a total cell number of 3.25×10^7 . Cell number increased very little during the first hour, but logarithmically between hours 1 and 9, with a mean generation time of approximately 11 hours. The rate of increase declined thereafter, and cell number at 24 hours was 2.5 times that at the start.

P³² incorporation into the various phosphorus-containing fractions is given in

FIGURE 1.— P^{32} incorporation into phosphorus-containing fractions of cultured cells. Specific activity expressed as counts per minute per μ mole of phosphorus.

Figure 1. Active synthesis of all four fractions occurred in the cultured cells. Rapid initial incorporation of P^{32} into the acid-soluble fraction was observed, the specific activity reaching a maximum in 3 hours and declining thereafter to an equilibrium value which was maintained to the end of the experiment. Specific activity of the RNA fraction increased linearly during the first 3 hours, and then at a declining rate to the end of the experiment. In general, P^{32} incorporation into DNA paralleled the increase in cell number. No measurable incorporation of P^{32} into DNA was observed during the first hour of culture. Thereafter, the specific activity of the DNA fraction increased rapidly during the interval between the first and third hours, and at a slower rate to the end of the experiment.

Alkaline phosphatase: Changes observed in the specific activity of alkaline phosphatase during the development of Oregon R-EL2 flies are given in Figure 2. The level of activity observed in eggs was very low, barely above the lower limit of sensitivity of the assay. This level was increased about tenfold in third instar larvae, exhibited a decline thereafter, finally reaching a level in adults which was approximately 3 to 4 times that present in eggs. The results obtained with *ry*² and *ma-l* were essentially the same as those obtained with Oregon R-EL2.

Embryonic cells freshly isolated from eggs exhibited a specific activity of about

FIGURE 2.—Changes in alkaline phosphatase activity during normal development of Oregon R-EL2 flies at 22°C.

FIGURE 3.—Changes in alcohol dehydrogenase activity during normal development of Oregon R-EL2 flies at 22°C.

0.002 units/mg protein, a value which was comparable to that exhibited by the intact eggs. Such cells were cultured for 20 hours in complete H-5 medium and in complete H-5 supplemented with various concentrations of β -glycerophosphate to investigate changes in alkaline phosphatase activity. Cell number increased about 1.6 times during this period (from 7.5×10^6 to 12.4×10^6) both in the absence and presence of β -glycerophosphate. No significant changes in enzyme level were observed during the culture period, and the presence of β -glycerophosphate in concentrations ranging from $1 \times 10^{-2}M$ to $3 \times 10^{-2}M$ had no significant effects.

The possibility that the capacity of embryonic cells to synthesize alkaline phosphatase might change during more prolonged culture was investigated by growing the cells in complete H-5 medium for variable periods of time extending to 6 days, and then transferring them to medium supplemented with $2 \times 10^{-2}M$ β -glycerophosphate for an additional 20 hours. The results suggested a gradual decline in the ability to produce even the very low level of enzyme present initially, and no change was observed in the lack of responsiveness to β -glycerophosphate.

This lack of responsiveness was also exhibited with a second enzyme substrate, phenylphosphate. Cells cultured for 20 hours in the presence of $2 \times 10^{-2}M$ or $5 \times 10^{-2}M$ phenylphosphate showed no significant change in alkaline phosphatase activity. A similar lack of responsiveness was observed in experiments where cells were cultured for 20 hours in basal H-5 (containing no calf serum) supplemented with β -glycerophosphate, indicating that this result is not attributable to degradation of substrate by serum phosphatases.

Alcohol dehydrogenase: Changes observed in the specific activity of alcohol dehydrogenase during the development of Oregon R-EL2 flies are given in Figure 3. No significant enzyme activity was detectable in eggs, but third instar larvae exhibited a level of enzyme activity about 25 times above the lower limit of sensitivity of the assay. This was almost doubled in 2-day pupae, and declined to about the larval level in adults. The results obtained with *ry^s* and *ma-l* were essentially the same, except that adults from these stocks exhibited about 20 to 25% more enzyme per mg of protein than did Oregon R-EL2 adults.

Embryonic cells freshly isolated from eggs exhibited a specific activity of 1.2×10^{-2} units/mg protein, a value much above that exhibited by intact eggs. It would appear that all or most of the enzyme in eggs is intracellular, and that the failure to detect enzyme in intact eggs is attributable to dilution by yolk and other extracellular constituents.

When embryonic cells were cultured in complete H-5 medium supplemented with various concentrations of ethanol, significant effects on alcohol dehydrogenase levels were observed (Figure 4). In the absence of ethanol the specific activity of the enzyme remained virtually constant during the first 42 hours of culture and then declined significantly to about one-third of its original level at 66 hours. In the presence of ethanol, ranging in concentration from 1% to 5%, the specific activity of the enzyme increased during the first 42 hours of culture, reaching a level which was about double the original value. The level tended to decline thereafter, but precipitously in the case of 5% ethanol where it fell to control values at 66 hours.

Effects of ethanol on cell multiplication are given in Figure 5. Concentrations of 1% and 2.5% may have been slightly stimulatory to cell multiplication, while a concentration of 5% had a small deleterious effect after 48 hours of culture.

FIGURE 4.—Alcohol dehydrogenase activity of embryonic Oregon R-EL2 cells cultured in 5 ml of complete H-5 medium containing indicated concentrations of ethanol. T-30 flasks. 30°C. Initial cell number approximately 3×10^5 per ml.

FIGURE 5.—Growth of embryonic Oregon R-EL2 cells in T-30 flasks containing 5 ml of complete H-5 medium with the indicated concentrations of ethanol. 30°C.

Microscopic examination of the cells disclosed no marked cytological effects of ethanol, except for inhibition of the clumping of large cells described in previous publications (HORIKAWA and FOX 1964; HORIKAWA *et al.* 1966). Blebbing was not observed.

Since these experiments suggested that cultured embryonic cells respond to the presence of ethanol by exhibiting elevated alcohol dehydrogenase levels, further studies were performed of the effects of the addition or removal of ethanol at various times during the primary culture period. Cultures were established in complete H-5 medium and in complete H-5 supplemented with 2.5% ethanol. Cells from the former were transferred to complete H-5 supplemented with 2.5% ethanol after 20 and 42 hours of culture. Cells from the latter were transferred to complete H-5 without ethanol after 20 and 42 hours. Control cultures were maintained in complete H-5 or in complete H-5 containing 2.5% ethanol through the whole duration of the experiment, but with transfers to fresh medium at 20 and 42 hours. Enzyme levels were determined at the beginning of the experiment and after 20, 42, and 66 hours of culture. The results are given in Figure 6.

In summary, cells transferred to medium containing ethanol after a period of culture in the absence of ethanol responded thereafter in a manner parallel to that exhibited by cells which were cultured continuously in ethanol. Cells transferred to medium lacking ethanol after a period of culture in the presence of ethanol responded thereafter in a manner parallel to that exhibited by cells which were cultured continuously without ethanol.

Methyl alcohol (1.5%) also stimulated alcohol dehydrogenase activity, although less effectively than did ethanol, while isopropanol (2.5%) depressed alcohol dehydrogenase activities after the first 20 hours of culture. Cells cultured in the presence of ethanol exhibited normal alkaline phosphatase and xanthine dehydrogenase activities.

FIGURE 6.—Response of cultured embryonic Oregon R-EL2 cells to the addition or removal of ethanol. Cells cultured at 30°C in T-30 flasks containing 5 ml of complete H-5 (—) or 5 ml of complete H-5 with 2.5% ethanol (---). Initial cell number approximately 3×10^5 per ml. (a) Continuous culture in the absence of ethanol. (b) Culture in the presence of ethanol for 20 hours, followed by transfer to medium lacking ethanol. (c) Culture in the absence of ethanol for 42 hours, followed by transfer to medium containing ethanol. (d) Culture in the absence of ethanol for 20 hours, followed by transfer to medium with ethanol. (e) Culture in the presence of ethanol for 42 hours, followed by transfer to medium lacking ethanol. (f) Continuous culture in the presence of ethanol.

FIGURE 7.—Changes in xanthine dehydrogenase activity during normal development of Oregon R-EL2 flies at 22°C.

Xanthine dehydrogenase: Changes observed in the specific activity of xanthine dehydrogenase during the development of Oregon R-EL2 flies are given in Figure 7. Eggs exhibited significant but low activity, while the enzyme activity present in third instar larvae was about 13 times above the lower limit of sensitivity of the assay. There was a slight increase above this level in pupae, and the larval level was doubled in adults. No detectable xanthine dehydrogenase activity was found in *ry²* or *ma-l* at any time during development.

Freshly isolated embryonic cells exhibited a specific activity of 2.2×10^{-4} units/mg protein, a value similar to that exhibited by intact eggs. Such cells were cultured for 20 hours in the presence and absence of hypoxanthine. Cell number increased 1.8 times during this period (from 8.2×10^6 to 14.7×10^6) in both cases. Enzyme activities observed at the end of the culture period are given in Table 1. The specific activity of cells cultured in the absence of hypoxanthine was significantly above the level exhibited when they were freshly isolated, and the presence of hypoxanthine in the culture medium had a depressing effect on enzyme activity. This depression became more pronounced with increasing concentrations of hypoxanthine. A similar depression of enzyme activity was exhibited by cells cultured for 7 days in the presence of 2×10^{-4} M hypoxanthine. Xanthine and uric acid, the intermediate and final products of xanthine dehydrogenase activity, had the same effect (Table 1). The levels of alcohol dehydrogenase and alkaline phosphatase were not affected by the presence of any of these compounds in the medium.

Since xanthine dehydrogenase is present in cultured cells, it seemed possible that the depressing effect of hypoxanthine and xanthine resulted from their conversion to uric acid, i.e. that the active agent was always the latter, acting either by inhibition of the enzyme or by repression of enzyme synthesis. To

TABLE 1

Effects of hypoxanthine, xanthine, and uric acid on the xanthine dehydrogenase activity of cultured embryonic Oregon R-EL2 cells

Addition to medium	Concentration (M)	Xanthine dehydrogenase (units/mg protein $\times 10^4$)
Hypoxanthine	0	5.9
	8×10^{-6}	5.6
	5×10^{-5}	4.1
	2×10^{-4}	1.0*
Xanthine	0	6.5
	5×10^{-5}	4.5
	1×10^{-4}	2.7
	2×10^{-4}	1.2*
Uric acid	0	7.4
	5×10^{-5}	5.0
	1×10^{-4}	2.1
	2×10^{-4}	0.7*

Cells were cultured for 20 hours at 30°C in T-30 flasks containing 5 ml of complete H-5 with indicated additions. Initial cell number approximately 1.6×10^6 per ml.

* Values not significantly different from zero.

TABLE 2

Reversal by dilution of the uric acid inhibition of xanthine dehydrogenase

Dilution factor	Xanthine dehydrogenase (units $\times 10^4$)	
	Observed	Expected*
1	63	63
2	45	31.5
4	26	15.75
8	15	7.875
16	9	3.9375

0.7 ml of a larval enzyme extract was mixed with 0.7 ml of $3.2 \times 10^{-3}M$ uric acid in 0.1M Tris buffer and incubated at 4°C for 2 hours. A series of doubling dilutions of the mixture were made with 0.1M Tris buffer and assayed in the usual fashion. pH was maintained at 8.0 in all cases.

* Calculated as activity of undiluted mixture divided by dilution factor.

test this possibility, the affect of uric acid on the activity of enzyme extracted from third instar larvae was studied.

Uric acid proved to be a powerful dissociable inhibitor of larval xanthine dehydrogenase. For example, a preparation which exhibited a specific activity of 26.9×10^{-4} units/mg protein in the absence of uric acid was inhibited to the extent of 41.6% by $4 \times 10^{-4}M$ uric acid, and to the extent of 79.2% by $3.2 \times 10^{-3}M$ uric acid. The inhibitory effect was completely reversed by dialysis against buffer in both cases. The dissociable character of the inhibition was also demonstrated by dilution experiments (Table 2). Dilution of mixtures of enzyme and uric acid resulted in a reduction of activity which was smaller than that expected for a non-dissociable complex, i.e. the reduction in activity was not proportional to the dilution factor.

Studies of the kinetics of this inhibition served to characterize it as purely noncompetitive (DIXON and WEBB 1964). When the activity of larval extracts were assayed at several different uric acid concentrations at each of two different hypoxanthine concentrations, the results were as given in Figure 8. A plot of the reciprocal of activity against uric acid concentration yielded a straight line for each substrate concentration with a point of intersection on the base-line. Tests performed with several different uric acid concentrations at each of several different hypoxanthine concentrations demonstrated that relative inhibition was independent of substrate concentration. This is illustrated in Figure 9, where the data are plotted according to the method of WILSON (1949). All of the points fit a single straight line with unit intercept on the ordinate, as is expected of purely noncompetitive inhibition. The equilibrium constant for the union of uric acid with enzyme, K_i , is $2.19 \pm 0.03 \times 10^{-4}M$ as calculated by the method of HUNTER and DOWNS (1945).

These results suggest that the depression of xanthine dehydrogenase activity in embryonic cells cultured in the presence of hypoxanthine, xanthine, or uric acid is attributable at least in part to inhibition by the latter. If this is the case, uric acid should be associated with the enzyme extracted from such cells. The low enzyme concentration in these extracts prevented a direct test of this pre-

FIGURE 8.—Kinetics of the inhibition of larval xanthine dehydrogenase by uric acid. An extract of third instar Oregon R-EL2 larvae was assayed at two substrate concentrations ($5 \times 10^{-5}M$ and $1 \times 10^{-4}M$ hypoxanthine) in the presence of the indicated concentrations of uric acid. v = units of xanthine dehydrogenase.

FIGURE 9.—Relative inhibition of larval xanthine dehydrogenase by uric acid. Three extracts of third instar Oregon R-EL2 larvae were prepared and assayed in the presence of the indicated concentrations of uric acid. One was assayed with $1 \times 10^{-4}M$ hypoxanthine, the second was assayed with $5 \times 10^{-5}M$ hypoxanthine, and the third was assayed with $2.5 \times 10^{-5}M$ hypoxanthine. v_0 = xanthine dehydrogenase units in the absence of uric acid. v_i = xanthine dehydrogenase units in the presence of uric acid.

TABLE 3

Kinetics of xanthine dehydrogenase extracted from Oregon R-EL2 embryonic cells cultured in the presence and absence of hypoxanthine

Cells cultured in	Concentration of hypoxanthine used in assay (M)	Enzyme activity (units/mg protein $\times 10^4$)
Complete H-5	2.63×10^{-5}	4.3
	5.26×10^{-5}	5.7
	10.00×10^{-5}	6.3
Complete H-5 + 2×10^{-4} M hypoxanthine	2.63×10^{-5}	3.1
	5.26×10^{-5}	3.4
	10.00×10^{-5}	3.1

Cells were cultured for 20 hours at 30°C in T-30 flasks containing 5 ml of medium. Initial cell number approximately 1.6×10^6 per ml. Extracts were prepared and assayed as indicated.

diction, but it was possible to study the dependence of enzyme activity on substrate concentration. For this purpose, cells were cultured in the presence and absence of hypoxanthine, enzyme extracts were prepared, and the extracts were assayed with several different substrate concentrations. The results are given in Table 3. The reaction velocity exhibited by extracts from cells grown in the absence of hypoxanthine showed typical hyperbolic dependency on substrate concentration, fitting the Michaelis-Menten relationship. Extracts from cells grown in the presence of hypoxanthine, on the other hand, exhibited no significant change in reaction velocity when substrate concentration was increased from 2.63×10^{-5} M to 10^{-4} M. This result would be expected if uric acid was associated with the enzyme in such extracts.

DISCUSSION

Working with nine strains of primary fibroblasts derived from human skin, Cox and PONTECORVO (1961) observed marked increases in alkaline phosphatase activity when β -glycerophosphate or phenylphosphate were added to the medium. The same compounds did not produce elevations of alkaline phosphatase activity in epithelial cell strains, but the latter did respond to treatment with the hydrocortisone analogue, prednisolone (Cox and MACLEOD 1961, 1962). The molecular mechanisms were not elucidated. In the case of cultured embryonic cells of *Drosophila*, the failure to respond could be a reflection either of impermeability to organic monophosphates or to fundamental noninducibility of alkaline phosphatase synthesis. It is possible, of course, that there was a response on the part of one or more of the electrophoretically distinguishable alkaline phosphatases present in *D. melanogaster* but that this was missed in the assay for over-all activity. For the time being, however, it may be taken that embryonic cells of *Drosophila* do not respond to the presence of substrate in the medium by an increased synthesis of alkaline phosphatase.

By way of contrast, these cells exhibit marked responses to the addition and removal of ethanol. The increases and decreases in alcohol dehydrogenase activity

observed in this case resemble the increases and decreases of cholinesterase activity in cultures of chick embryonic cells in response to the addition or removal of acetylcholine in the medium (BURKHALTER *et al.* 1957). Since the levels of xanthine dehydrogenase and alkaline phosphatase are not affected, since the increases and decreases in alcohol dehydrogenase are changes in specific activity, and since there is a rapid response to the addition and removal of substrate, there is an excellent possibility that this is a system which exhibits true substrate induction of enzyme synthesis (JACOB and MONOD 1961). Alternatively, it is possible that ethanol in the medium promotes the aggregation of enzymatically inactive monomers to form active dimers or multimers, or that it serves to stabilize the enzyme.

It should be noted that the external concentrations of ethanol used in these experiments were distinctly unphysiologic, ranging from 0.17M to 0.86M, but that the growth rate of the cells was affected only at the highest concentration. The only other observable effect was on the spontaneous aggregation normally exhibited by the large cell type in such cultures. It is quite likely that the cell membrane of the cells is able to limit the increase in internal concentration of ethanol even in the face of high external concentrations.

The depressing effect of hypoxanthine, xanthine, and uric acid on the specific activity of xanthine dehydrogenase resembles the depression of glutamine synthetase by glutamine in cultured human cells (DEMARS 1958). In the present case, the depression seems to be attributable in major part to inhibition of enzyme activity by its product, uric acid, rather than to an effect on the rate of enzyme synthesis, although a more critical demonstration of the presence of uric acid in cellular extracts will be necessary. This is particularly urgent, since the passage of the extracts over Norite-A would have been expected to remove uric acid. It should be noted that the inhibition of xanthine dehydrogenase by uric acid has been confirmed in the laboratory of H. S. FORREST (personal communication).

We have previously reported the presence of two cell types, large and small, in primary cultures of the sort used in the present investigation (HORIKAWA and Fox 1964). The former, which resemble yolk cells and constitute 47% of the initial population, divide only once on the average during a culture period of 7 days and decline to 29% of the population at the end of such a period. The latter, which resemble blastoderm cells, exhibit an average generation time of 25 to 30 hours during log phase growth, and are responsible for the maintenance of long-term cultures (HORIKAWA, LING, and Fox 1966). Owing to its slow division rate the large cell type gradually declines in frequency in long-term cultures, and virtually disappears after 450 days.

DOLFINI and GOTTARDI (1966) have reported very early onset of mass chromosomal abnormalities in primary cultures. Critical examination of their published figures indicates that their analysis dealt selectively with the large cell type. They have probably explained, therefore, the slow growth rate of this cell type, but have dealt with cells that are of minor importance in the dynamics of the cultures. Chromosome number in small cells does not depart from normal in primary cultures.

The present work does not attempt to ascribe the observed responses specifically to either cell type. In addition, no attempt was made to distinguish the different isozymes known to exist for each of the enzymes studied. Since this is the case, and since there is no assurance that the behavior of embryonic cells *in vitro* is comparable to that of their counterparts *in vivo*, the present studies can be regarded only as a most modest start toward answering the question posed in the introduction to this paper.

Nevertheless, the results show that in embryonic cells *in vitro* each of the three enzymes responds differently to the presence of its substrate. This suggests that different mechanisms are responsible for the specific appearance of each of the three enzymes at a particular time and place during development. Detailed studies of each system will be needed to elucidate these mechanisms.

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SUMMARY

Active RNA and DNA synthesis occurs in primary cultures of embryonic cells from *D. melanogaster*. Such cells, when cultured in basal medium, exhibit very low levels of alkaline phosphatase, alcohol dehydrogenase, and xanthine dehydrogenase activities. During normal development, alkaline phosphatase activity reaches a peak in third instar larvae. The addition of enzyme substrates, β -glycerophosphate or phenylphosphate, to the medium has no effect on the alkaline phosphatase levels exhibited by embryonic cells in primary cultures. Alcohol dehydrogenase activity reaches a peak during the pupal period. The addition of ethanol to the medium in appropriate concentrations has no marked effect on the growth rate of embryonic cells, but results in a doubling of alcohol dehydrogenase activity. Methanol has a similar, although less marked, effect. The response of cultured cells to the addition of ethanol is rapid, and removal of ethanol from the medium results in a rapid decline in alcohol dehydrogenase activity. Alkaline phosphatase and xanthine dehydrogenase levels are not affected. Xanthine dehydrogenase reaches a peak in adults. The addition of hypoxanthine, xanthine or uric acid to the medium results in a significant depression of xanthine dehydrogenase activity extractable from cultured embryonic cells. Studies with larval enzyme demonstrate that uric acid is a noncompetitive inhibitor of xanthine dehydrogenase, suggesting that the depression of enzyme activity in embryonic cells by hypoxanthine and xanthine results from their conversion to uric acid and enzyme inhibition.

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