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Alkaline Phosphatase Modulation by Osmolality Changes During the Growth Cycle of KB Cell Cultures[1] (38857)

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Previous studies have shown that KB cells, originally derived from a human nasopharyngeal carcinoma (1) display very high alkaline phosphatase (orthophosphoric monoester phosphohydrolase EC 3.1.3.1) activity (2). In these cells the enzyme is composed of a large heat-labile and a small heat-stable component (2), which also differ in their susceptibility to inhibition by inorganic phosphate (3). By contrast to the increase in enzyme activity occurring when HeLa S3 (a cell line with low alkaline phosphatase) is grown in media made hyperosmolar with NaCl or in media supplemented with prednisolone (4), culturing KB cells under these conditions causes a striking reduction in the specific activity of alkaline phosphatase without affecting the activity of several other enzymes (5). This quantitative change is accompanied by alterations in the proportion of the heat-labile and heat-stable enzyme components. This report concerns the modulation of alkaline phosphatase activity and thermostability by alterations in the osmolality of the culture medium during the growth cycle of KB cells. Evidence will be presented showing that in cells growing in hyperosmolar medium, total enzyme activity increases only during the initial 24 hr after the change in osmolality but that the heat-stable activity increases 7-fold within 48 hr. By contrast, in KB cells growing in standard medium, total alkaline phosphatase activity increases progressively during a 6-day growth cycle with the proportion of the heat-stable activity remaining constant throughout.

Materials and Methods. KB cells were obtained from Microbiological Associates, Inc., Bethesda, MD. The cells were grown as monolayers in milk dilution bottles using Puck's Medium supplemented with 10% undialyzed human serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). The cells were checked periodically for mycoplasmas and were found to be free of contamination. The osmolality of the culture medium was increased by the addition of appropriate amounts of NaCl (from an autoclaved 3 *M* stock solution) 24 or 48 hr after cell transfer. The culture technique, harvesting, and subsequent handling of the cells were as previously described (5). Alkaline phosphatase activity was measured by the hydrolysis of *p*-nitrophenyl phosphate (6) using 2-amino-2-methyl-1-propanol-HCl buffer (pH 10.6) at 38°. Duplicate assays agreed with less than 10% variation. Specific activity was expressed as micromoles of *p*-nitrophenol liberated in 30 min at 38° per mg of protein, the latter determined according to Lowry *et al.* (7).

Thermostability of alkaline phosphatase was investigated by incubating triplicate aliquots of 0.05 ml of cell-free sonicates at 56° with 0.1 ml of 1 *M* 2-amino-2-methyl-1-propanol-HCl buffer (pH 10.6). After incubation for various lengths of time, tubes were transferred to 4° and the residual activity was subsequently measured at 38° by the addition of 0.1 ml of 0.016 *M* *p*-nitrophenyl phosphate containing 2 m*M* $MgCl_2$. The proportion of activity remaining was calculated from the controls preincubated with buffer at 4.° The heat-stable activity in relation to the total specific activity was computed by extrapolation to zero time of curves depicting the inactivation rates (5). In other studies, enzyme preparations were preincubated with buffer at 56° for 20 min and the results were expressed as percentage of activity remaining, calculated from controls kept at 4°.

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TABLE I. EFFECT OF GROWTH IN HYPEROSMOLAR MEDIUM ON THE ALKALINE PHOSPHATE OF KB CELLS[a].

	Regular Medium			Hyperosmolar Medium	
Hours of growth	Total specific activity	Stable specific activity	Hours after adding NaCl	Total specific activity	Stable specific activity
48	26	3.7	0	27	3.6
72	33	8.0	24	50	15.0
96	96	12.0	48	52	26.0
120	110	14.0	72	53	30.0
144	116	12.0	96	48	29.0

[a] Cells suspended in saline F were inoculated into 20 culture bottles containing medium N16. After 48 hr two sets of duplicate cultures were harvested and 30 m*M* NaCl were added to eight bottles (hyperosmolar medium). The remaining eight bottles were left undisturbed (regular medium). Duplicate cultures of both sets were harvested at 24-hr intervals. Enzyme activity was determined in duplicate and related to cell protein. Heat-stable activity was computed as indicated in text.

Results. The alkaline phosphatase activity of KB cells, like that of other heteroploid cells of human origin is "self-inducible," i.e., the specific activity increases progressively during a 6- to 8-day growth cycle (2). In order to obtain information on the effects of hyperosmolality as a function of time, alkaline phosphatase activity and thermostability were ascertained at 24-hr intervals in KB cells growing under standard conditions and in hyperosmolar medium. As can be seen in Table I, the total specific activity of control cultures (302 mOsm) increased rapidly from 26 at 48 hr to 98 at 96 hr, before reaching 116 by 144 hr. By contrast, the total activity of cells growing in hyperosmolar medium only rose from 27 at 48 hr (when the osmolality of the medium was increased from 302 mOsm to 362 through the addition of 30 m*M* NaCl) to 50 at 72 hr (24 hr in hyperosmolar medium), but did not increase any further during the next 72 hr. The total cell protein content of the experimental cultures was essentially identical to that of their respective controls growing in regular medium.

By preincubating cell-free enzyme preparations from each culture at 56° in 0.67 *M* 2-amino-2-methyl-1-propanol-HCl buffer (pH 10.6) for various lengths of time and by extrapolation to zero time of the curves depicting the slower inactivation rates, which represent the heat-stable enzyme component, it was possible to ascertain the specific activity of this component (5). As indicated in Table I, the proportion of heat-stable activity in relation to the total activity of cells growing in regular medium remained almost constant, increasing from 3.7 at 48 hr to a maximum of 14 at 120 hr. On the other hand, despite the fact that the total enzyme activity of KB cells growing in hyperosmolar medium had leveled off by 48 hr after the addition of NaCl, the proportion of heat-stable activity continued to rise. A 7-fold increase in the specific activity of the heat-stable component, from 3.6 to 26, was noted during the initial 48 hr after the osmolality change and toward the end of the growth cycle, most of the enzymes was heat-stable.

Incubation for up to 48 hr of intact cells attached to glass in NaCl-supplemented saline F (which does not support cell growth) or the addition of NaCl to cell-free enzyme preparations does not affect alkaline phosphatase activity or thermostability (5). These findings suggest that the reduction in activity and the transition in thermostability require cell multiplication. Hence, it was of importance to determine the influence of hyperosmolality on the change in specific activity and thermostability in relation to the time of addition of NaCl during the growth cycle of KB cells. For this purpose the osmolality of the medium of a series of cultures was increased from 302 mOsm to 382 mOsm with 40 m*M* NaCl at 24-hr intervals. Cultures to which no additions were made served as controls. All cultures were confluent when harvested after a growth cycle of 168 hr. After the determination of total specific activity, triplicate samples from each dupli-

TABLE II. EFFECT OF TIME IN HYPEROSMOLAR MEDIUM ON ALKALINE PHOSPHATASE ACTIVITY AND THERMOSTABILITY OF KB CELLS.[a]

Hours in hyperosmolar medium	Total specific activity	% Heat-stable activity
0 (control)	116	6.6
24	106	15.9
48	84	17.0
72	78	26.0
96	64	51.5
120	48	66.4

[a] Cells suspended in saline F were inoculated into regular N16 medium. Forty millimolar NaCl was added to duplicate cultures 48 hr after cell transfer and then every 24 hr. No addition was made to controls. All cultures were harvested after 168 hr. Total specific activity was determined as indicated in text. Percentage heat-stable activity refers to alkaline phosphatase activity remaining after incubation at 56° for 20 min with 0.67 *M* 2-amino-2-methyl-1-propanol-HCl buffer (pH 10.6).

cate culture were tested for thermostability. In Table II it can be seen that there was an inverse relationship between total specific activity and the length of growth in hyperosmolar medium. Thus, the enzyme activity of KB cells grown for 48 hr in regular medium and 120 hr in hyperosmolar conditions was 41% that of cells grown in regular medium and 55% the activity of cells initially grown for 120 hr in standard medium followed by 48 hr in hyperosmolar medium. In regard to thermostability, there was an almost linear relationship between the length of time the cells were grown in hyperosmolar medium and the proportion of heat-stable activity. Under the experimental conditions used, the alkaline phosphatase of cells initially grown for 48 hr in standard medium followed by 120 hr in hyperosmolar medium lost 34% of the activity, whereas with cells grown for 144 hr in normal medium and 24 hr in the altered medium, 84% of the activity was destroyed. By comparison, 93% of the activity was inactivated in enzyme preparations from control cultures grown for 168 hr in regular N16 medium (Table II).

Additional support to the notion that cell multiplication is required for the transition in thermostability was provided by experiments in which the enzyme of control cultures and of cells growing in medium made hyperosmolar at various stages of the growth cycle was compared at 24-hr intervals. As can be seen in Fig. 1, the percentage of activity remaining after preincubation at 56° for the enzyme of control cultures (0 hr in hyperosmolar medium) showed no significant variation. It oscillated between 6.4% and 7.0%. The effect of hyperosmolality on the stability of alkaline phosphatase was discernible in all instances within 24 hr, and the proportion of heat-stable activity increased as a function of cell growth. With cells grown for 24 hr in regular medium followed by 24 hr in hyperosmolar conditions, 26% of the activity remained after 20 min at 56°. When cultures from the same set were allowed to grow for an additional 48 hr, 59% of the activity remained. However, the percentage of residual activity in preparations from cells grown for 120 hr in hyperosmolar medium was only 65%, indicating a leveling off which was also noted with the enzyme from cultures made hyperosmolar late in the growth cycle.

Discussion. The study of alkaline phosphatase as a marker in investigations of malignant cells has received new impetus as witnessed by some recent reports. Thus, it has been shown that embryonal mouse carcinoma (teratoma) cells have activity levels 5- to 100-fold higher than somatic cell lines (8). Sela and Sachs (9) have demonstrated that virally and chemically transformed hamster cells display markedly lower alkaline phosphatase activity than normal cells and that prednisolone induces increased levels of activity in virally transformed but not in chemically transformed cells (10). Among human cells, prednisolone and/or hyperosmolality induce increased activity only in certain heteroploid lines (e.g., HeLa S3, T24) possessing low, heat-stable alkaline phosphatase (4, 11). In the case of KB cells which have two catalytically similar alkaline phosphatases differing in their thermostability, hyperosmolality causes induction of increased levels of activity of the small heat-stable enzyme component while simultaneously suppressing the large, heat-labile component. Thus, the over-all response is

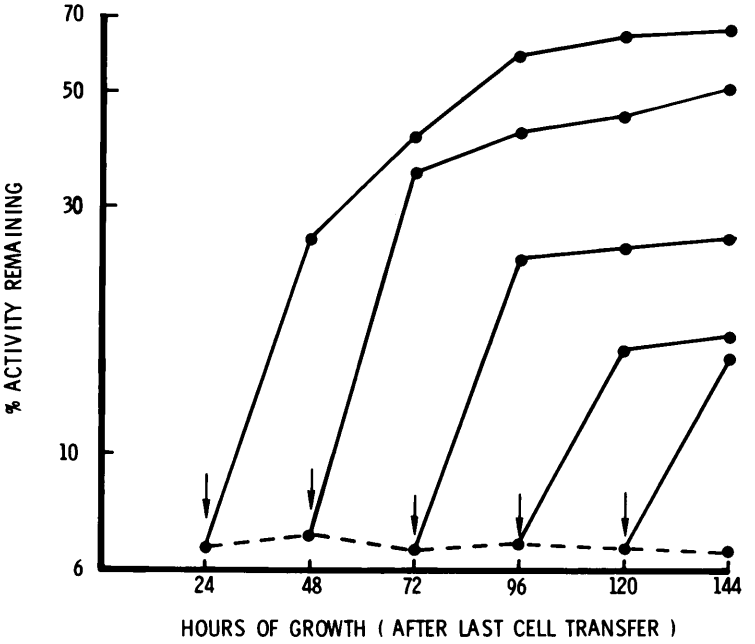

FIG. 1. Effect of hyperosmolality on alkaline phosphatase thermostability. KB cells suspended in saline F were inoculated into regular N16 medium, 24 hr after cell transfer, and thereafter at 24-hr intervals, one set of duplicate control cultures (- - - -) was harvested and 40 m*M* NaCl was added to another set of duplicate cultures (arrows). These experimental cultures (——) were also harvested at 24-hr intervals. The harvested cells were washed thrice with 0.15 *M* NaCl and stored at −20°. Thermostability of alkaline phosphatase activity was determined by preincubating triplicate 0.05-ml portions of cell-free enzyme preparations with 0.1 ml of 1.0 *M* 2-amino-2-methyl-1-propanol-HCl buffer (pH 10.6) at 56° for 20 min. Percentage of activity remaining was computed from preparations preincubated with buffer at 4°.

manifested by a reduction in total enzyme activity.

The transition of the total alkaline phosphatase activity from predominantly heat-labile into relatively heat-stable under the influence of hyperosmolality is seen only with growing cultures; no changes in activity and/or thermostability are noted when NaCl is added to nongrowing KB cells or to cell-free enzyme preparations. The effect of hyperosmolality is discernible within 24 hr after the addition of NaCl to the nutrient environment. Although the total specific activity in cells grown under hyperosmolar conditions remains constant for 72 hr, the proportion of heat-stable activity increases progressively to the extent that most of the activity (>65%) is thermostable at the end of a growth cycle. It is interesting to note that an increase in heat-stable activity is also detectable in cultures made hyperosmolar toward the end of the growth cycle. This would indicate that when KB cells are reaching confluency, they still respond to increased osmolality with an alkaline phosphatase of lower specific activity but with enhanced thermostability.

Although the mechanism involved in the osmolality-dependent modulation of alkaline phosphatase in KB cells is not understood, it appears to be different than that regulating the hormonal induction of tyrosine aminotransferase in cultured rat hepatoma cells (12). In the latter system the increased activity is accounted for by an increase in the rate of synthesis of tyrosine aminotransferase molecules which have the same kinetic and physicochemical properties as the basal enzyme (13). On the other hand, studies with HeLa cells suggest that the corticosteroid-dependent induction of increased levels of alkaline phosphatase activity is not due to a change in the rate of synthesis or degradation of the enzyme. It

appears that the steroid triggers the synthesis of a modifier molecule which interacts with alkaline phosphatase producing an enzyme with enhanced catalytic efficiency which, except for an increased first-order constant for the decomposition of the enzyme-substrate complex (k_3), is otherwise indistinguishable from the base-level enzyme (14, 15). Because the heat-stable enzyme component of KB cells responds to the same stimuli as the heat-stable alkaline phosphatase as HeLa S3 cells (4), the suggestion has been made (5) that similar mechanisms are operative and that in both cell lines this enzyme represents the product of the same genetic locus, whereas the heat-labile component in KB cells is the product of a different locus.

Summary. Growing KB cells in hyperosmolar medium causes a reduction in total alkaline phosphatase activity associated with a decrease in the proportion of the heat-labile and an increase of the heat-stable enzyme components. In standard medium enzyme activity increases progressively during a 6-day growth cycle and the proportion of heat-stable activity remains constant. In hyperosmolar medium, activity increases only during the initial 24 hr after the change in osmolality and then levels off, but the heat-stable alkaline phosphatase activity increases 7-fold within 48 hr. The transition in thermostability is discernible 24 hr after increasing the osmolality of the medium and toward the end of a growth cycle most of the activity is heat-stable.

1. Eagle, H., Proc. Soc. Exp. Biol. Med. **89,** 362 (1955).
2. Herz, F., Kaplan, E., and Fineman, E. L., Biochim. Biophys. Acta **304,** 660 (1973).
3. Herz, F., In Vitro **10,** 63 (1974).
4. Nitowsky, H. M., Herz, F., and Geller, S., Biochem. Biophys. Res. Commun. **12,** 293 (1963).
5. Herz, F., Arch. Biochem. Biophys. **158,** 225 (1973).
6. Herz, F., and Nitowsky, H. M., Arch. Biochem. Biophys. **96,** 506 (1962).
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
8. Bernstine, E. G., Hooper, M. L., Grandchamp, S., and Ephrussi, B., Proc. Nat. Acad. Sci. USA **70,** 3899 (1973).
9. Sela, B.-A., and Sachs, L., Fed. Eur. Biochem. Soc. Lett. **30,** 100 (1973).
10. Sela, B.-A., and Sachs, L., J. Cell. Physiol. **83,** 27 (1974).
11. Herz, F., Barlebo, H., and Koss, L. G., Cancer Res. **34,** 1943 (1974).
12. Tomkins, G. M., Levinson, B. B., Baxter, J. D., and Dethlefsen, L., Nature New Biol. **239,** 9 (1972).
13. Gelehrter, T. D., Emanuel, J. R., and Spencer, C. J., J. Biol. Chem. **247,** 6197 (1972).
14. Cox, R. P., Elson, N. A., Tu, S.-H., and Griffin, M. J., J. Mol. Biol. **58,** 197 (1971).
15. Ghosh, N. K., Rukenstein, A., Baltimore, R., and Cox, R. P., Biochim. Biophys. Acta **286,** 175 (1972).

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