INDUCTION OF ALKALINE PHOSPHATASE IN DISPERSED CELL CULTURES BY CHANGES IN OSMOLARITY[1]

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During the course of studies on the mechanisms regulating synthesis of alkaline phosphatase in dispersed cell cultures, a striking induction of enzyme activity was observed in response to increase in the osmolarity of the nutrient medium. Although the relation of this finding to induction of enzyme activity by adrenal glucocorticoids (Cox and MacLeod, 1961) remains obscure, these results may provide an alternative explanation for certain effects ascribed to substrate induction (Maio and De Carli, 1963). In addition, they emphasize the importance of distinguishing between effects related to changes in osmolarity or other mechanisms in studies of the responses of mammalian cells to alterations of the nutrient environment.

Methods: The cell lines employed and the methods for growth and preparation of cells for enzyme assay have been described previously (Nitowsky and Herz, 1961). Various inorganic salts, sucrose, or prednisolone 21-phosphate were added 24 or 48 hours after cell transfer. Alkaline phosphatase activity of sonicates was measured with p-nitrophenyl phosphate in 2-amino-2-methyl-1-propanol (AMP) buffer, pH 10.6, and specific activity was expressed as μmoles p-nitrophenol liberated in 30 minutes per mg protein. The ratio of the specific activity of the experimentally treated to the control culture was referred to as the "induction ratio".

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Results: The induction of alkaline phosphatase in human cell lines after growth for 6 days in Puck's medium containing added inorganic salts is shown in Table 1. Both cell lines showed an increase in enzyme activity with added NaCl or KCl. In some experiments, an increase in enzyme activity was discernable with additions of as little as 5 mM NaCl. With the sulfates and, in other experiments, the dibasic phosphates, similar changes in enzyme activity were observed. Qualitatively similar alterations were noted with added $CaCl_2$ and LiCl, although the toxic effects of these salts, as manifested by some inhibition of cell proliferation and protein synthesis, were greater than with equimolar concentrations of NaCl or KCl.

Table 1. EFFECT OF INCREASE IN OSMOLARITY ON ALKALINE PHOSPHATASE ACTIVITY

Cell Line	Addition (mM)		Estimated Osmolarity (mOsm/L)	Specific Activity	Induction Ratio	Cell Protein (mg/flask-6 day growth)
	Control		302	0.46	---	1.25
Zimmer liver clone 2A	NaCl	14	330	0.72	1.6	1.16
		28	358	1.38	3.0	1.13
		42	386	2.02	4.4	1.18
		56	414	3.10	6.7	1.18
	Control		302	4.06	---	1.15
	NaCl	20	342	9.87	2.4	1.26
		40	382	15.55	3.8	1.07
		80	462	28.91	7.1	0.66
Zimmer liver clone 4A	KCl	20	342	9.84	2.4	1.25
		40	382	18.28	4.5	0.95
	Na_2SO_4	20	362	11.87	2.9	1.12
		40	422	18.14	4.4	0.95
	K_2SO_4	20	362	11.40	2.8	1.07
		40	422	16.22	3.9	0.46

The similar results with various inorganic salts indicated a lack of specificity of either cationic or anionic species in eliciting this response. The induction of enzyme activity following addition of isosmotic quantities of sucrose, which is not metabolized by mammalian cells in vitro (Eagle et al., 1958), showed that the changes observed were related to an osmotic rather than an ionic effect (Table 2). The simultaneous addition of NaCl and sucrose

yielded an additive effect, as did various combinations of Na or K salts.

Table 2. EFFECT OF VARIOUS AGENTS ON ALKALINE PHOSPHATASE ACTIVITY OF HELA S_3

Addition*	Specific Activity	Induction Ratio	Cell Protein (mg/flask)
Control	0.33	---	1.24
NaCl	0.85	2.6	1.22
Sucrose	1.16	3.5	1.20
NaCl + Sucrose	2.93	8.9	1.00
Prednisolone	0.95	2.9	1.33
Prednisolone + NaCl	1.80	5.4	1.19
Prednisolone + Sucrose	2.06	6.2	1.25
NaCl + Puromycin	0.36	1.1	0.73
Sucrose + Puromycin	0.19	0.6	0.55

* Substances added in following concentrations: NaCl 42 mM, sucrose 82 mM, prednisolone 1.0 μg/ml, puromycin 0.5 μg/ml.

Increase in alkaline phosphatase was observed 24 hours after addition of NaCl, and the effect was maximal after 72 hours. Following return to regular growth medium, the specific activity fell progressively, suggesting dilution of enzyme activity by cell multiplication. Incubation of mixtures of cell-free extracts excluded the possibility that the differences were due to activators in the salt-treated cultures or to inhibitors in the controls. No changes in activity were observed after pre-incubation of non-growing cell suspensions with salts, and there was no effect of 80 mM NaCl on the enzyme reaction. Acid phosphatase showed no changes in specific activity following growth of cells with added NaCl. No impairment in synthesis of cell protein was noted up to increments of 80 mM NaCl, although Stubblefield and Mueller (1960) reported some inhibition of cell proliferation at lower salt concentrations.

Studies were carried out on the induction of alkaline phosphatase in response to the combined stimuli of increase in osmolarity and addition of prednisolone. At a concentration of the latter of 1.0 μg/ml, at which the response to the steroid was maximal, the addition of NaCl or sucrose resulted in an even greater enzyme induction (Table 2), and the effects of the various combinations of agents were additive. These findings suggested that different

mechanisms were involved in enzyme induction by adrenal glucocorticoids and increase in osmolarity. The inhibition of the response to NaCl or sucrose addition by puromycin was similar to that observed in phosphatase induction with steroids (Nitowsky and Herz, 1963) and supported the conclusion that de novo protein synthesis was involved in these changes.

Since the change in alkaline phosphatase of low activity cell lines with adrenal steroids differed from that of high activity lines (Nitowsky and Herz, 1963), a similar comparison was made of the changes in phosphatase in response to increase in osmolarity (Table 3). In general, low activity cell lines responded both to steroid and to added NaCl by induction of alkaline phosphatase. Although enzyme induction by prednisolone was maximal and characteristic for each cell line at concentrations of 0.5 to 1.0 μg/ml, the induction ratio rose with increase in osmolarity.

Table 3. INDUCTION OF ALKALINE PHOSPHATASE OF VARIOUS CELL LINES BY STEROID AND SALT

	SPECIFIC ACTIVITY		
Low Activity Cell Lines	Control	Prednisolone (0.5 μg/ml)	NaCl (40 mM)
HeLa S_3	0.22	0.70	0.85
Zimmer liver clone 2A	0.46	2.10	2.02
Chang liver clone 2	0.46	2.96	2.24
Chang liver clone 6A	1.68	4.87	8.72
High Activity Cell Lines			
Detroit 6	79.7 (4*)	36.2 (58)	144.8 (10)
Heart	77.1 (5)	73.0 (23)	96.5 (12)
Intestine	33.1 (2)	22.1 (20)	49.5 (10)
H. Ep. II	39.9 (92)	60.8 (85)	50.0 (87)

* Percent residual activity after incubation for 10 min. at 56°C in 0.75 M AMP buffer, pH 10.6.

With high activity cell lines (Table 3), the changes in response to added steroid or NaCl were qualitatively different. Although a striking decrease in enzyme activity was seen after growth with prednisolone, except with H. Ep. II, the addition of 40 mM NaCl produced an increase in phosphatase activity. These changes supported the contention that the mechanisms underlying induction by adrenal steroids and changes in osmolarity differed.

The alkaline phosphatase of low activity cell lines is heat-stable (Herz and Nitowsky, 1962) and no change in this property was observed in steroid or osmotically induced cultures. In contrast, the enzyme in high activity cell lines exhibits intracellular molecular heterogeneity, consisting of a heat-labile and a heat-stable fraction. In response to added NaCl there was an increase in heat-stable activity in the latter group (Table 3). These changes resembled those of prednisolone-treated cultures, although the increase was more marked following steroid induction. Unlike the steroid effect, however, there was also a greater heat-labile phosphatase activity following increase in osmolarity, and this contributed to an increase in the total specific activity. The H. Ep. II cell line, which differs from other high activity lines in possessing a heat-stable enzyme, showed an increase in total alkaline phosphatase activity with steroid as well as with added salt.

Chang liver clone 8A also differed from other high activity cell lines in that total phosphatase activity diminished following growth with added NaCl as well as with prednisolone (Table 4). In both instances the heat-stable fraction increased. Following the simultaneous addition of both salt and steroid an equivalent reduction in total phosphatase activity occurred. However, there was a greater increase in the heat-stable fraction, representing the sum of the contribution from prednisolone and NaCl when added independently.

In contrast to the findings with established lines, recently isolated fibroblast cell cultures from skin (grown in minimal Eagle's medium with 20% human serum and 5% bovine embryo extract ultrafiltrate) had low alkaline phosphatase activity but showed no change in response to steroid or to increase in osmolarity. The fact that these cell lines showed practically no heat-stable activity may provide an explanation for the differences in response to these stimuli.

Little information is available concerning the cellular metabolic responses to alterations in osmolarity. Studies of the salt requirements of

Table 4. EFFECT OF SALT AND STEROID ON HEAT-STABLE PHOSPHATASE OF CHANG LIVER CLONE 8A

Addition*	Specific Activity	Heat-Stable Fraction (%)**	Specific Activity of Heat-Stable Fraction
Control	79.9	3	2.4
Prednisolone	47.6	17	8.3
NaCl	45.0	16	7.2
Prednisolone + NaCl	49.2	40	19.6

* Prednisolone and NaCl added in concentrations of 0.5 µg/ml and 40 mM respectively.

** Obtained by extrapolation to zero time of first order rate of inactivation of heat-stable component at 56°C in AMP buffer.

mammalian cell cultures heretofore have been restricted largely to the parameters of cell increase and gross morphologic changes (Eagle, 1956) and have been considered in terms of specific ionic rather than osmotic effects. Recent studies (Stubblefield and Mueller, 1960) have revealed changes in composition and rates of glucose utilization and lactate production of HeLa cells in response to high concentrations of NaCl in the nutrient medium. However, ionic and osmotic effects were not differentiated. Inhibition of phagocytosis and the respiratory and glycolytic activities associated with this process following the addition of various inorganic salts or glucose to guinea pig polymorphonuclear leucocytes were ascribed specifically to the effects of hyperosmolarity (Sbarra et al., 1963).

The induction of alkaline phosphatase is the first example of an enzymatic alteration in mammalian cells in response to increase in osmolarity. While this alteration differs in certain respects from phosphatase induction by adrenal glucocorticoids, it may provide a basis for studies of possible mechanisms involved in the steroid effect. Furthermore, changes in metabolic and enzymatic activity of cells in vitro in response to changes of osmolarity may serve as a model for studies of the homeostatic responses to similar pathologic events in vivo.

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