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*INDUCTION OF ALKALINE PHOSPHATASE BY SUBSTRATES IN ESTABLISHED CULTURES OF CELLS FROM INDIVIDUAL HUMAN DONORS**

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Many microbial systems of inducible and repressible enzyme synthesis are known.[1–4] A few examples of inductive and repressive effects have been described in cells of higher organisms in tissue culture.[5–9] In most of these cases, the difference between maximum activity, induced or repressed, and control activity is not very great. The present communication describes the induction in established cultures of human skin fibroblasts of alkaline phosphatase activity (presumably *via* enzyme synthesis) by putative substrates of the enzyme. An advantage of this alkaline phosphatase system is that cells grown in the absence of inducer have little or no detectable enzyme activity, while induced cultures show very substantial activity, 30 to 50 fold greater than the minimum detectable by our methods.

That alkaline phosphatase synthesis in homeotherm cells might be based on an inducer-repressor system is suggested by embryological evidence.[10, 11] During early embryonic development in the chicken, all cells contain alkaline phosphatase.[10] Enzyme activity decreases in most tissues to undetectable levels during the intermediate stages of development. Later, it reappears in the organs and tissues which in the adult normally contain the enzyme.[10,11] These include the intestinal epithelium, proximal tubule of the kidney, osteoblasts, granulocytes, liver cells, and a few others. However, the enzyme is "absent" in most other tissues such as the fibroblasts of the skin, though it may appear under special circumstances, such as healing skin wounds, and in certain tumors derived from tissues which do not otherwise show it.[12–14]

The presence of alkaline phosphatase activity in all cells of the early embryo

and its restricted or conditional location in the adult suggests that it is based on an inducible system. This is known to be so in microorganisms,[2] in explants of amphibian,[15] avian, and mammalian embryonic intestine,[11] and in human granulocytes *in vivo*.[16] In microorganisms, alkaline phosphatase synthesis is induced when the concentration of one product of its activity, inorganic phosphate, is reduced in the medium.[2] In explants of embryonic amphibian, avian, and mammalian intestine, alkaline phosphatase synthesis is induced at an earlier stage than normal by either adrenal glucocorticoids or certain presumed substrates of the enzyme.[11, 15] Human granulocyte alkaline phosphatase is markedly increased in patients treated with hydrocortisone.[16] Cox and MacLeod[9] have shown that a hydrocortisone analogue, prednisolone, induces alkaline phosphatase activity in some but not all established human "epithelial" cell lines. The present communication describes the substrate induction of alkaline phosphatase activity in established human skin "fibroblastic" cultures from individual healthy donors.

Methods.—Cell cultures used for the present work are from strains available in this laboratory and originally established either by trypsinizing with a modified Younger technique[17] or by explanting small pieces of tissue. All but one strain are derived from foreskins of children. The exception (HR) is from the forearm of a healthy female donor. They are uncloned, grown on glass surfaces in Waymouth's medium with 10 per cent heated calf serum, and kept in culture by successive trypsinizations. For each experiment, the cells of a strain were pooled from several bottles, and the same cell numbers from this pool were inoculated into each of the bottles required for the experiment. The only known difference between bottles of one experiment was the addition of putative substrate or special medium. The substrates used were disodium phenylorthophosphate (Hopkin & Williams Ltd.) or disodium β glycerophosphate (Eastman Kodak Ltd.).

Cells were harvested by trypsinization and counted in triplicate in a Levy hemocytometer. The cells were twice washed in saline and lysed in 1% aqueous sodium deoxycholate at a concentration of 5 million cells/ml. Alkaline and acid phosphatase were determined by a modification of Huggins and Talalay phenolphthalein phosphate method.[18] Magnesium chloride at a final concentration of 0.003 *M* was added to the substrate. To reduce the number of cells required, a semimicro method using 0.1 cc of deoxycholate lysate and 0.4 cc of substrate was used. Incubation was at 37°C and pH 9.75 for 4 hr in order to increase the accuracy in cases where low levels of alkaline phosphatase were present. Colorimetric determinations were made immediately after adding 0.4 cc glycine buffer to the reactants, employing a Unicam SP.600 spectrophotometer with semimicro cuvette at 540 mμ. alkaline phosphatase results are expressed as mg of phenolphthalein liberated by 5 million cells during 4 hr incubation at 37°C. The amount of phenolphthalein released was proportional to alkaline phosphatase at concentrations of 0.2 mg per ml phenolphthalein or greater in a 4-hr period. Below this concentration of phenolphthalein, enzyme activity was not proportional to enzyme concentration. This sets the lower limit of accurate measurement of enzyme activity in our experiments.

Experimental Results.—Nine individual human skin cell strains were studied from 6 to 15 months after the cultures were established. All nine cell strains lacked alkaline phosphatase activity when cultured in the usual manner (Table 1) (i.e., with inocula of 200,000 or more cells in a 6-oz medicine bottle with 10-ml medium, so that subculturing is required every 7 to 12 days). As previously reported,[9] prednisolone (phosphate or hemisuccinate) at concentrations of 0.5 μg/ml did not influence the alkaline phosphatase activity of the skin fibroblast strains studied (Table 1). On the other hand, the results of the present experiments show that high alkaline phosphatase activity is induced by both of two methods in seven out of nine fibroblast strains (Table 1). One of the two remaining strains "Wil" is induced by one method but not by the other, and the other strain "Fio" is not induced by either.

TABLE 1

ALKALINE PHOSPHATASE ACTIVITY AMONG CULTURES OF ESTABLISHED SKIN FIBROBLASTS FROM INDIVIDUAL DONORS

Donor	Duration of cultivation, months	Chromosome number	Alkaline Phosphatase Activity: Without induction	With prednisolone	With phenyl-phosphate	In own old medium
Cla	8	46	−	−	+	+
Gey	8	46	−	−	+	+
Kel	8	...	−	−	+	+
Mas	8	46	−	−	+	+
Kut	8	...	−	−	+	+
H.R.	6	...	−	...	+	+
Hal	8	...	−	...	+	+
Wil	15	...	−	...	+	−*
Fio	12	...	−	−	−	−

+ Alkaline phosphatase activity present.
− Alkaline phosphatase activity absent.
... Not tested.
* Induced by old conditioned medium from an inducible strain (Mas).

TABLE 2

INDUCTION OF ALKALINE PHOSPHATASE ACTIVITY IN HUMAN FIBROBLASTS GROWN IN THE PRESENCE OF PHENYLPHOSPHATE FOR 7 TO 15 DAYS

Cell strain	Number of experiments	Alkaline Phosphatase Activity*: Cells ($5 \cdot 10^6$) grown without phenylphosphate	Cells ($5 \cdot 10^6$) grown with phenylphosphate
Mas†	7	<0.02	0.40
Cla†	4	<0.02	0.40
Kel†	1	<0.02	0.33
Gey†	2	<0.02	0.14
Kut†	1	<0.02	0.40
H.R.†	1	<0.02	0.25
Hal	1	<0.02	0.27
Wil	1	<0.02	0.20
Fio†	2	<0.02	<0.02

* Milligram of phenolphthalein released by a deoxycholate lysate containing $5 \cdot 10^6$ cells. Incubation at 37° for 4 hr.
† Other experiments using qualitative methods gave similar results.

The first method is the growth of cell cultures in the presence of organic monophosphates (Table 2). Disodium phenylmonophosphate in concentrations in the growth medium ranging from 0.5 mM to 5 mM induces alkaline phosphatase activity in eight of the nine fibroblast strains. Higher concentrations of phenylphosphate inhibit cell multiplication. As seen in Tables 1 and 2, one of the nine strains (Fio) was not induced by growth with phenylphosphate. Figure 1 shows the result of an experiment with the human skin fibroblast strain "Mas." Alkaline phosphatase activity of cultures grown in the presence of 5 mM phenylphosphate is 40 fold greater than the minimum amount detectable by our method. Replicate cultures grown in the absence of inducer for the same time have alkaline phosphatase activity below the sensitivity of the method (0.02

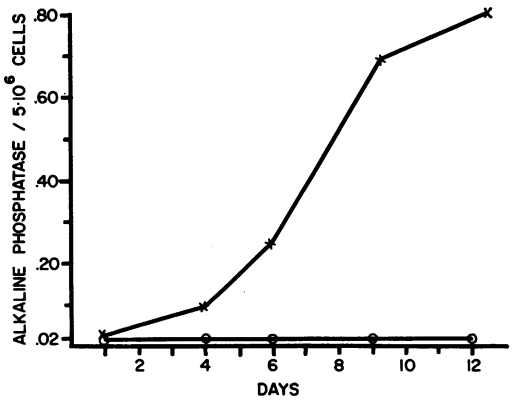

Fig. 1.—Alkaline phosphatase activity of replicate cultures grown with (×) and without (○) 5 mM phenylphosphate (strain Mas).

TABLE 3

COMPARISON OF ALKALINE PHOSPHATASE ACTIVITY IN HUMAN SKIN FIBROBLASTS GROWN FOR NINE DAYS IN THE PRESENCE OF EITHER PHENYLPHOSPHATE OR β GLYCEROPHOSPHATE

Cell strain	Alkaline Phosphatase Activity*		
	No inducer	β glycerophosphate (5 mM)	Phenylphosphate (5 mM)
Mas	<0.02	0.09	0.590
Cla	<0.02	0.05	0.200
Kel	<0.02	0.04	0.130

* See footnote to Table 2.

mg of phenolphthalein). β glycerophosphate disodium salt in equimolar concentrations was less effective than phenylphosphate as an inducer of alkaline phosphatase synthesis (Table 3). Acid phosphatase activity is present in all the cell strains tested. It is not altered by growth in medium with added phenylphosphate.

Unlike bacterial alkaline phosphatase,[2] that of human skin fibroblast cultures is insensitive to the inorganic phosphate concentration of the medium. This concentration is about 1.5 mM in the medium used; reduction to 0.05 mM does not induce activity, and increase up to 5.0 mM does not repress induction by the substrate.

Experiments on HeLa cultures indicate that in only one (Henle) of three HeLa lines (Henle, NIH, and MBA) could phenylphosphate induce alkaline phosphatase activity. Even in the Henle line, induction was possible only with very high concentrations of phenylphosphate (20 mM or greater) and the induced activity was only three to five fold over that of the controls. In all three of the HeLa lines studied, alkaline phosphatase activity was readily induced by prednisolone (0.5 μg/ml).[9]

The second method of alkaline phosphatase induction in human skin fibroblasts is to start a culture with a small inoculum and let the cells multiply to the maximum without changing medium. If 30–60 thousand cells are inoculated in a 6-oz medicine bottle with 15 ml of Waymouth medium containing 10 per cent calf serum, it is possible to keep the culture for at least 20 days without adding or changing medium. Seven of nine skin fibroblast strains developed alkaline phosphatase activity comparable to that following substrate induction when handled in this way (Table 4). Two of the strains (Fio and Wil) did not respond to this treatment. Induction by this not precisely controllable method was observed in about three-quarters of the experiments with the seven "self-inducible" fibroblast strains.

When cells of an inducible strain are inoculated in medium in which cells of the same or another "self-inducible" strain have grown for at least 20 days ("old conditioned medium"), alkaline phosphatase activity appears within 6 to 13 days in eight of nine cell strains. Table 5 shows the results of experiments on four fibroblast strains, two "self-inducible" (Mas and Kel) and two not inducible by "aging" (Wil and Fio). Using qualitative methods, the other five human fibroblast strains (Cla, Gey, Kut, Hal, and H.R.) were readily induced by "old conditioned medium." When cells from an inducible strain are seeded into 20- to 40-day-old medium from either one of the two negative cell strains (Fio or Wil), no alkaline phosphatase synthesis occurs. An observation of particular interest is strain "Wil" which does not develop activity by the "aging" procedure described and does not "condition" for other strains the medium in which it is grown but responds, nevertheless,

TABLE 4

INDUCTION OF ALKALINE PHOSPHATASE ACTIVITY IN HUMAN FIBROBLASTS GROWN WITHOUT CHANGE IN WAYMOUTH MEDIUM FOR 20 TO 40 DAYS

Cell strain	Age of culture, days	Alkaline phosphatase activity*
Mas†	30	0.50
Cla†	23	0.32
Kel†	30	0.15
Gey†	30	0.50
Kut†	24	0.30
H.R.	27	0.40
Hal	33	0.20
Wil†	22	<0.02
	30	<0.02
	40	<0.02
Fio†	19	<0.02
	22	<0.02
	34	<0.02
	40	<0.02

* See footnote to Table 2.
† Other experiments using qualitative methods gave similar results.

TABLE 5

INDUCTION OF ALKALINE PHOSPHATASE ACTIVITY IN HUMAN FIBROBLASTS GROWN IN "OLD MEDIUM" CONDITIONED BY INDUCIBLE CELL STRAINS

Cell strain	Duration of growth, days	Alkaline Phosphatase Activity*	
		Cells ($5 \cdot 10^6$) grown in fresh Waymouth	Cells ($5 \cdot 10^6$) grown in "old conditioned" Waymouth
Mas†	7	<0.02	0.375
	8	<0.02	0.250
	10	<0.02	0.400
Kel†	6	<0.02	0.500
Wil	13	<0.02	0.400
Fio†	11	<0.02	<0.02
	13	<0.02	<0.02

* See footnote to Table 2.
† Other experiments using qualitative methods gave similar results.

to "old conditioned medium" obtained from a "self-inducible" strain (Table 5).

Dilution of "old conditioned medium" with an equal volume of fresh Waymouth medium destroys its ability to induce alkaline phosphatase in the inducible strains. An aqueous extract prepared by freezing and thawing a suspension of induced cells when added to cultures did not induce.

Discussion.—The work reported demonstrates induction of alkaline phosphatase in established cultures of human skin fibroblasts by a substrate of the enzyme. The change in enzyme activity is high in comparison to that reported for other inducible or repressible mammalian systems.[5-9] The substances (phenylphosphate and β glycerophosphate) which we have found to be effective for the induction of alkaline phosphatase are substrates *in vitro* for this enzyme as well as for acid phosphatase. They have, however, no effect on the acid phosphatase activity of skin fibroblasts. In animal tissues, alkaline phosphatase is probably a heterogeneous group of enzymes and its *in vivo* enzymatic function and substrate(s) are unknown. In cultured human cells, the apparent inability of inorganic phosphate to repress alkaline phosphatase induction even at high concentrations suggests that the enzymes may not act as hydrolases *in vivo*.

There is a striking difference in the induction of alkaline phosphatase between established cultures of human "epithelial" and "fibroblastic" cells. Most of the former, as shown by Cox and MacLeod,[9] are inducible by a hormone analogue

(prednisolone) but not by low concentrations of substrate. The latter are not inducible by the hormone but respond very effectively to the substrate. Out of nine of the skin fibroblast strains studied, eight were inducible by phenylphosphate. The exception was strain "Fio." Whether this difference reflects variation between donors, variation between the types of cells established in culture, or variation which occurred during culture has not yet been determined. The inability to induce alkaline phosphatase in mammalian cells by means of substrates reported by Klein[7] may have been due either to the cell lines studied or to the concentration of substrate used. As we have shown, some HeLa lines are inducible only by very high concentrations of substrates, others are not inducible, and most skin fibroblasts are inducible by much lower concentrations. The inducibility of skin fibroblast cultures by substrate and the development of activity during wound healing *in vivo*[12] may be related.

When seven of nine skin fibroblast strains are grown from a small inoculum in a relatively large amount of medium for 20 or more days, high alkaline phosphatase activity usually appears. The medium ("old conditioned medium") in which this "self-induction" occurred is able to induce alkaline phosphatase activity promptly in cells of the strain grown in it as well as of other "self-inducible" strains. "Old conditioned medium" can also induce activity promptly in strain "Wil" which is not able to "self-induce" and the old medium of which is not able to induce other strains. Finally, strain "Fio" is not inducible by aging, growth in "old conditioned medium," or substrate. These findings suggest that strain "Wil" is unable to "condition" medium but is able to develop alkaline phosphatase activity, while strain "Fio" is unable to do either. The conditioning of medium may involve either production of one or more inducing substances or removal of one or more inhibitors present in fresh medium: it is perhaps significant that a small dilution of "old conditioned medium" with a fresh one leads to loss of activity.

The conditioning of medium, the determination of restricted distribution of constitutive alkaline phosphatase among different tissues, and the development of the enzyme under special conditions (e.g., wound healing and tumors) are likely to be interrelated processes with an important bearing on differentiation, normal and abnormal.

Summary.—Induction of alkaline phosphatase activity in established (6 to 15 months) human skin "fibroblastic" strains from different donors can be obtained by two methods. One method is by adding substrates of the enzyme to cultures. The other is less consistent and less well defined. It consists of growing a small cell inoculum in a relatively large amount of medium for a long period (20 or more days). The "old" medium is "conditioned" by the induced cells and will induce other susceptible strains. There are variations between human fibroblast strains in their response to either of these methods.

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*THE CHEMICAL AND MUTAGENIC SPECIFICITY OF HYDROXYLAMINE**

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The hereditary changes that convert one base pair (or base) of nucleic acid into another one have been divided into two classes—transitions and transversions.[1] This paper is concerned with the further subdivision of mutagenic transitions. For this purpose, the specific mutagen hydroxylamine[2] (HA) has been employed, which, for the normal DNA bases, reacts with cytosine (C) and 5-hydroxymethylcytosine (HMC) but not or only little with 5-methylcytosine (MeC) or thymine (T). The reaction with C has been examined in detail, since it is apparently responsible for the mutagenic effect of HA. Other bases, e.g., 5-bromouracil (BU), also react strongly with HA, but for BU-containing phages this effect is mainly lethal. In contrast to HA, the chemically similar agent hydrazine reacts much more with T than with C and is much less mutagenic.

The induction of reverse mutations by HA shows a clear bipartition into highly and little inducible phage T4 *r*II mutants; this indicates which base pair is present at the mutant site.

Abbreviations used: A = adenine, HMC = 5-hydroxymethylcytosine, C = cytosine, G = guanine, T = thymine. AP = 2-aminopurine, BU = 5-bromouracil, EES = ethylethane sulfonate, HA = hydroxylamine. CR = cytosine riboside, CdR = cytosine deoxyriboside, etc. dHMP = 5-hydroxymethylcytosine-deoxyribotide. G-C = guanine-cytosine pair in DNA, where C stands for any one of the cytosines.

Material and Methods.—The phage T4 *r*II mutants, bacteriological media, and genetic methods have been described previously.[2-5]