gain. Our results in mice are substantially in agreement with those results in rats.

The findings of these studies do not preclude the possible effect of radiation on temporary delays in DNA synthesis and mitosis soon after irradiation and the existence of at least some delay in these events is supported by the observation by Cole and Rosen(8) of decreased mitotic counts at 48 hours after unilateral nephrectomy when radiation was given 2 weeks before or 3 hours after nephrectomy.

The specific pattern of DNA synthesis and mitosis after irradiation and nephrectomy as well as the effect of humoral agents and diet in retarding or stimulating hypertrophy and hyperplasia must be investigated.

*Summary.* Three hours following unilateral nephrectomy weanling female CF-1 mice were subjected to local abdominal or total body exposure to either 500 or 600 r. Other groups were given 1350 r to half the abdomen with and without prior nephrectomy. Significant decreases in renal weight gain compared to controls were obtained in the 600 r total body group and in the locally irradiated animals given 1350 r. When the renal weight changes are compared on the basis of the ratio of renal weight to body weight in per cent, an increase from .64% to .95% is seen in animals subjected to nephrectomy alone. The final value in all of the irradiated and nephrectomized groups was similar, ranging from .93% to 1.0%. The results suggest that irradiation in these dose ranges does not exert a direct inhibitory effect on renal weight increase but it limits body weight gain which influences kidney weight.

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Received June 21, 1965. P.S.E.B.M., 1966, v121.

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# Effects of Cations on Activity and Thermostability of Alkaline Phosphatase in HeLa Cells.* (30726)

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Induction of alkaline phosphatase in HeLa cells by glucocorticoids first observed by Cox and McLeod(1) has stimulated considerable interest in this enzyme. Several papers have appeared dealing with the purification and physicochemical characteristics of the tissue culture alkaline phosphatase(2,3) and with the specificity of the steroid effect(4,5,6). In some of its properties, *e.g.*, molecular heterogeneity and susceptibility to inhibition by chelating agents, tissue culture alkaline phosphatase resembles the corresponding nonspecific alkaline phosphatases from bacteria(7) and from mammalian tissues(8).

Because $Zn^{++}$ and $Mg^{++}$ are involved in the activation of both animal and bacterial alkaline phosphatases(7,8), we have attempted to determine whether a similar requirement for cations may be demonstrated in preparations derived from HeLa cells. Stimulation of alkaline phosphatase by $Mg^{++}$ has been indicated in experiments of Cox and McLeod(9), but the effects of this ion on thermal stability have not been studied in detail. Moreover, no role has yet been

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* This investigation was supported in part by USPHS General Research Support Grant 1 SO1 FR-05373.

ascribed to Zn$^{++}$ with regard to its stimulatory effect on the tissue culture enzyme.

*Materials and methods. Cells and media.* HeLa cells adapted to calf serum were purchased from Microbiological Associates, Bethesda, Md. They were grown as monolayer cultures in Eagle's medium supplemented with 5% calf serum. For induction of alkaline phosphatase, prednisolone was added to the cultures at the level of 0.5 $\mu$g per ml. The cell cultures were incubated at 36.5°C and were harvested after four days.

*Enzyme preparations.* Deoxycholate lysates, prepared according to the method of Cox and McLeod(9), were used as the source of enzyme. In several experiments cell sonicates were compared with deoxycholate preparations, and in all such cases the results were essentially identical.

*Alkaline phosphatase assay.* The enzyme activity was determined colorimetrically by following hydrolysis of *p*-nitrophenylphosphate according to method previously described(4). Unless otherwise specified, 2-amino-2-methyl-1-propanol buffer was substituted for glycine buffer and Mg$^{++}$ was omitted from the reaction mixture.

*Results. Effect of Zn$^{++}$ on the activity of alkaline phosphatase.* Lower activity of alkaline phosphatase in the glycine buffer as compared to the activity in the 2-amino-2-methyl-1-propanol or veronal buffers suggested the possibility that glycine might chelate cation(s) required for the enzyme activity. Since Zn$^{++}$ is known to be a prosthetic group of kidney(8) and *E. coli*(7) alkaline phosphatase, graded amounts of ZnSO$_4$ were added together with the enzyme preparations to the glycine or 2-amino-2-methyl-1-propanol buffers. After 15 minutes of equilibration at 37°C in a water bath, substrate (*p*-nitrophenylphosphate) was added and the reaction was allowed to proceed for 30 minutes. The results of one such experiment are shown in Table I. In glycine buffer the activity was very low, but it increased with increasing the Zn$^{++}$ concentration up to a level of 10$^{-4}$ M. In contrast, Zn$^{++}$ was either inhibitory or without effect, depending on its concentration, if the reaction was carried out in the presence of 2-amino-2-methyl-1-propanol buffer. As shown in Table II, attempts to reverse the inhibition due to glycine by the addition of cations other than Zn$^{++}$ were unsuccessful.

TABLE I. Effect of Zn$^{++}$ on Inhibition of Alkaline Phosphatase Activity by Glycine.

| | Specific activity* of alkaline phosphatase at pH 9.1 | |
|---|---|---|
| Concentration of Zn$^{++}$ (molar) | Glycine buffer (.33 M) | 2-amino-2-methyl-1-propanol buffer (.66 M) |
| 0 | 1.7 | 18.0 |
| $10^{-6}$ | 2.8 | 18.0 |
| $5 \times 10^{-6}$ | 5.3 | 17.8 |
| $10^{-5}$ | 6.0 | 17.8 |
| $5 \times 10^{-5}$ | 8.1 | 16.7 |
| $10^{-4}$ | 9.0 | 15.1 |
| $5 \times 10^{-4}$ | 8.5 | 10.6 |
| $10^{-3}$ | 8.3 | 7.0 |

Conditions: Reaction mixture contained 1 M substrate and an aliquot of enzyme preparation (equivalent to 50-100 $\mu$g of protein per ml) suspended in one of the indicated buffers.

* Specific activity = No. of micromoles of *p*-nitrophenol released in 2 hr at 37°C per mg of protein.

In order further to substantiate these results, 5 ml aliquots of deoxycholate lysates were dialyzed at 0°C against 200 volumes of either (a) 0.05% EDTA (6 changes every 24 hours) or (b) 0.1 M glycine buffer pH 9.1 (3 changes every 24 hours) followed by 0.05% EDTA (3 changes every 24 hours). Both preparations were further dialyzed against 3 changes of double-distilled water and then were assayed for alkaline phospha-

TABLE II. Effect of Cations on Activity of Alkaline Phosphatase in Presence of Glycine Buffer (0.66 M, pH 9.1).

| | Concentration (molar) | | |
|---|---|---|---|
| Cation | $10^{-6}$ | $10^{-5}$ | $10^{-4}$ |
| | | Specific activity* | |
| Fe$^{+++}$ | 2.5 | 2.1 | 3.0 |
| Fe$^{++}$ | 2.9 | 2.8 | 3.0 |
| Hg$^{++}$ | 2.0 | 2.0 | .8 |
| Co$^{++}$ | 2.1 | 2.3 | 3.2 |
| Pb$^{++}$ | 3.2 | 2.8 | 1.2 |
| Mn$^{++}$ | 2.5 | 3.1 | 3.6 |
| Al$^{+++}$ | 2.4 | 2.2 | 3.3 |
| Cu$^{++}$ | 2.4 | 2.4 | 2.0 |
| Cd$^{++}$ | 2.0 | .8 | .8 |
| Zn$^{++}$ | 3.7 | 12.0 | 13.1 |

Conditions: Reaction mixture similar to that of Table I.

* Specific activity in the absence of added cations had a value of 2.0.

TABLE III. Partial Reactivation of Alkaline Phosphatase Activity in Dialyzed Deoxycholate Lysates from HeLa Cells.

| Method of dialysis | Concentration $Zn^{++}$ | Specific activity |
|---|---|---|
| EDTA | 0 | .57 |
| | $10^{-6}$ | .73 |
| | $10^{-5}$ | .81 |
| | $10^{-4}$ | 4.08 |
| Glycine followed by EDTA | 0 | .69 |
| | $10^{-6}$ | .79 |
| | $10^{-5}$ | 2.12 |
| | $10^{-4}$ | 1.29 |
| Non-dialyzed control | | 12.20 |

tase activity in 2-amino-2-methyl-1-propanol buffer at pH 9.1. As shown in Table III, prolonged dialysis of the lysates caused a marked drop in the specific activity of the enzyme. This loss of activity was partially restored by the addition of $Zn^{++}$ during the reaction. Subsequent experiments have shown that concentrations of $Zn^{++}$ higher than $10^{-4}$ M were inhibitory. $Mg^{++}$, at concentrations as high as $10^{-2}$ M, had no effect on alkaline phosphatase activity in deoxycholate lysates.

*Effects of $Mg^{++}$ and $Zn^{++}$ on thermal inactivation of alkaline phosphatase.* Inactivation of alkaline phosphatase was followed by heating deoxycholate lysates at 56°C in 1 M 2-amino-2-methyl-1-propanol buffer at pH 11.0. At appropriate time intervals the pH of the reaction mixture was lowered to 9.1 and the enzyme activity was determined. In a typical experiment (Fig. 1) $Mg^{++}$ ($10^{-2}$ M) was added to the reaction mixture either alone or together with $Zn^{++}$ ($10^{-4}$ M). The pattern of heat inactivation resembled data obtained by Herz and Nitowsky(2) indicating heterogeneity of the enzyme. In our case, however, difference in heat stability between the induced and non-induced enzyme preparations was small. Most significant was the effect of $Mg^{++}$ which markedly retarded thermal inactivation of alkaline phosphatase. $Zn^{++}$, alone, was without effect but was found to potentiate the effect of $Mg^{++}$. Addition of $Mg^{++}$ to the heat inactivated preparations did not restore the enzyme activity.

Screening of other cations for protection against heat inactivation has shown that ammonium ions might have an effect similar to that of $Mg^{++}$, delaying thermal inactivation of the HeLa cell alkaline phosphatase.

*Discussion.* No explanation is available concerning the glucocorticoid stimulation of alkaline phosphatase because of the lack of definite information about the physiological role of this enzyme in cell cultures.

Recently, Nitowsky and Herz(3) presented data indicating that the more heat-stable form of alkaline phosphatase is affected by the hormone. In confirmation of their results, we have found that the induced enzyme is inactivated by heat more slowly than the non-induced enzyme. However, in our work the retardation of heat inactivation in the presence of varying concentrations of $Mg^{++}$ was very significant. It is possible that the apparent heat stability of some cell culture preparations of alkaline phosphatase reflect the $Mg^{++}$ content of the enzyme molecule. This possibility is supported by the data of Garen and Levinthal(10) who found dialyzed preparations of *E. coli* alkaline phosphatase to be considerably more heat-labile than the undialyzed preparations.

Although $Zn^{++}$ may be a prosthetic group of the tissue culture alkaline phosphatase, we do not have data as yet to show positive correlation between $Zn^{++}$ content of the enzyme and its specific activity.

*Summary.* In deoxycholate lysates from

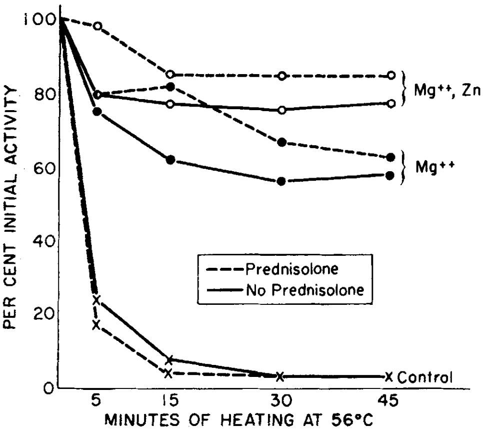

FIG. 1. Effect of $Mg^{++}$ and $Zn^{++}$ on thermal stability of alkaline phosphatase. Values for samples containing $Zn^{++}$ alone were almost identical with controls and were omitted from the graph for the sake of clarity.

HeLa cells $Zn^{++}$ reversed inhibition of alkaline phosphatase by glycine. Low concentrations of this cation were found to reactivate dialyzed preparations of the same enzyme. $Mg^{++}$ retarded thermal inactivation of alkaline phosphatase. Delay in heat inactivation was observed in alkaline phosphatase preparations from both prednisolone treated and control cultures of HeLa cells.

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Received June 28, 1965. P.S.E.B.M., 1966, v121.

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# Carcinogenic Effect of Cholesterol in Mice.* (30727)

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It was previously demonstrated that both egg white and egg yolk are carcinogenic in mice, but that their carcinogenicity is different. The white causes the development of a high incidence of lung adenocarcinoma and lymphosarcomas, while the egg yolk, in addition to these, causes the development of mammary cancer in the breeding females of the T.M. strain of mice(1). The mice of this strain on the control diet rarely develop mammary cancer. Moreover, it was found that the carcinogenic substances in egg are lipids; they are present in ether extract of whole egg and in alcohol extract of egg yolk(2). The carcinogenicity of these 2 extracts is not the same. Mice maintained on a diet supplemented with ether extract of whole egg develop a lower incidence of mammary cancer and a higher incidence of lung adenocarcinoma and lymphosarcoma than similar mice on a diet supplemented with alcohol extract of egg yolk. It is well known that some lipids are more soluble in alcohol while others are more soluble in ether. The conclusion was consequently drawn that there is a relationship between the types of malignancies and the composition of the lipids ingested by the mice. The results obtained with cholesterol alone and cholesterol and lard support the above point of view.

*Methods.* It was demonstrated that mice of the T.M. strain maintained on the Rockland rat diet supplemented with cholesterol and lard develop a relatively high incidence of malignancies (close to 50%). These malignancies are mainly lung adenocarcinomas and mammary cancer. Fifty per cent of the breeding females on the diet supplemented with cholesterol and lard developed breast cancer. In the present experiments mice of the T.M. strain were placed at the age of 4 weeks on the Rockland rat diet supplemented with cholesterol and lard (Group 1) or with cholesterol alone, no lard (Group 2). The cholesterol was given in the amount of 50 to 100 mg daily per group of 10 to 12 mice. For the mice of Group 1, one gram of lard and some sugar were mixed with the cholesterol. For those of Group 2, some sugar and a drop of water were mixed with the cholesterol. The mice ate the cholesterol and there was almost no waste. The mice of the control group were maintained on the Rockland rat diet only. The mice of the 3

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* Supported by USPHS Grant CA 04269 and by General Research Support Grant FR-5419-04.