Endocrine Relationships of Leukocyte Alkaline Phosphatase

By Fred Rosner and Stanley L. Lee

During the course of an investigation of leukocyte alkaline phosphatase activity in Down's syndrome, wide variations of this enzyme in the control population were noted. Other studies have also shown significant differences between normal subjects and patients with trisomy 21. These differences are consistent with the hypothesis that a gene for this enzyme is on chromosome 21. However, other factors also influence the phenotypic expression of this enzyme. The variability of leukocyte alkaline phosphatase activity with adrenal cortical function and elevation of levels of this enzyme in polycythemia vera and myeloid metaplasia are well-known. The present studies demonstrate a relationship between sex and leukocyte alkaline phosphatase. Attempts to show that this difference between men and women is due to differences in endocrine function rather than in genes, are described.

Materials and Methods

A. Sources

Blood was obtained from healthy children in a Well Baby Clinic and Guardian Home. Healthy adult control subjects consisted of physicians, medical students, nurses, laboratory technicians and other hospital personnel. Subjects who had any evidence of acute infection or fever were excluded. Hematologic abnormalities were ruled out by scanning peripheral blood smears. The numbers and distribution of subjects by sex, mean age and age range are shown in table 1.

B. Leukocyte Alkaline Phosphatase

Leukocyte alkaline phosphatase activity was determined by the following modification of previously described methods. Five to 8 ml of blood obtained by venipuncture were placed into a tube containing 3 ml of 6 per cent dextran and 1 ml of 4 per cent sodium citrate. The tube was inverted several times, placed in a rack and red blood cells were allowed to sediment at room temperature. The plasma layer was removed after 30 to 45 minutes and centrifuged at 500 rpm for 3 minutes. The sediment which contained white blood cells, platelets and some red blood cells was then washed three times with 0.85 per cent sodium chloride and resuspended in 1 ml of 0.85 per cent sodium chloride. Leukocyte counts were performed in duplicate on this suspension, 0.1 ml of which was then added to 1 ml of glycine buffered p-nitrophenyl-phosphate (pH = 9.3) as substrate. The length of time from venipuncture until the start of the incubation with substrate was kept under 1 hour. After incubation for exactly 30 minutes at 37 C., 10 ml of 0.02N sodium hydroxide were added, the tube stoppered and mixed by inversion and read...
LEUKOCYTE ALKALINE PHOSPHATASE

colorimetrically at 420 m\(\mu\) on a Coleman Junior Spectrophotometer. The alkaline phosphatase units corresponding to this reading were determined from a standard calibration curve. Concentrated hydrochloric acid 0.1 ml was then added, decolorizing the p-nitropheno! and leaving the color of the original white blood cell suspension, the optical density of which was then read at 420 m\(\mu\) and subtracted from the total optical density, giving the corrected phosphatase of the white cell suspension in "sigma"\(^{12}\) units. Results were then converted and recorded in milligrams of phosphorus liberated in 1 hour per 10\(^{10}\) polymorphonuclear neutrophilic leukocytes.

Each determination was performed in duplicate; pairs which did not agree within 10 per cent of each other were rejected.

Blood smears obtained from each patient at the time of venipuncture were stained by the Wright-Giemsa method. Differential white blood cell counts (200 cells) were performed on these smears, and the number of neutrophils was determined by multiplying percentage by the total count of the leukocyte suspension.

C. Saponization of Leukocytes

Nine volumes of a 1 per cent saponin solution were added to one volume of leukocyte suspension derived as previously described, and allowed to stand at 0 C. for 15 minutes. The resultant mixture was considered the total saponin lysate and phosphatase activity was then determined as described above. The total saponin lysate was then centrifuged at 8200g at 0 C. for 15 minutes and the supernatant was tested for phosphatase activity. The sediment or "nuclear pellet" was discarded except when it was "resaponized" as just described.

D. In Vitro Effect of Hormones on Leukocyte Alkaline Phosphatase Activity

The following pure crystalline hormones were prepared in varying concentrations by dissolving them in 95 per cent ethyl alcohol:

1. Estrogens
   - estrone
   - alpha estradiol
   - 17 beta estradiol
   - estradiol monobenzoate
   - progesterone
   - estrone-3-phosphate
   - estradiol-17-phosphate
   - estradiol-3,17-diphasate

2. Androgens
   - testosterone
   - testosterone propionate
   - testosterone undecylionate
   - testosterone valerate
   - epitestosterone propionate
   - testosterone cyclohexylpropionate
   - testosterone cyclohexylacetate
   - testosterone isobutyrate
   - testosterone trimethylacetate
   - dihydrotestosterone propionate

One-tenth ml. of white cell suspension was tested for phosphatase activity when 0.1 ml. of the respective hormone had previously been added to the incubation mixture. Since all hormones were dissolved in 95 per cent ethyl alcohol and the latter has some inhibitory effect on leukocyte alkaline phosphatase activity, the control used for all the in vitro measurements of effects of hormones was 0.1 ml. of 95 per cent ethyl alcohol (without hormones) added to the incubation mixture.

*Kindly supplied by the CIBA Pharmaceutical Company, Summit, New Jersey.
†Kindly supplied by Ing. B. Hogberg, AB. Leo, Halsingborg, Sweden.
‡Kindly supplied by Eli Lilly and Company, Indianapolis, Indiana.
E. In Vivo Effect of Hormones on Leukocyte Alkaline Phosphatase Activity

Ambulatory patients with carcinoma of the breast, either postmenopausal or surgically castrated, were studied. Leukocyte alkaline phosphatase activity was also tested in a control group of 10 patients receiving no therapy, a group of 6 patients receiving testosterone and 3 patients being treated with estrogens. Patients with carcinoma of the prostate receiving estrogens were also studied, using as controls patients with a similar diagnosis but not under treatment with estrogens.

F. Statistics

Logarithmic plots of the data and calculations were used throughout to achieve normality of distribution and equality of variance. Conversion to antilogarithmic values was made to facilitate comparison with values obtained by other workers. Snedocor's Statistical Methods was used as a reference.

RESULTS

A. Children

Leukocyte alkaline phosphatase levels were higher in children than in adults (figs. 1 and 2), as has been previously reported. For the 26 boys and 20 girls studied, the mean values were 66 and 70 mg. of phosphorus liberated per hour per $10^8$ polymorphonuclear leukocytes respectively (table 1). No significant difference between male and female children ($p > 0.5$) was found. Statistically significant regression (table 2) with age was found for both sexes (figs. 3 and 4).

B. Adults

Postpubertal men and women comprised this group. The women were all premenopausal. Men up to the age of 60 years were included. There was a very significant difference ($p < .001$) in leukocyte alkaline phosphatase activity between the sexes. The mean enzyme value of 74 healthy men was 23
LEUKOCYTE ALKALINE PHOSPHATASE

M A L E S

Fig. 2.—Distribution of leukocyte alkaline phosphatase values in normal male subjects.

mg. of phosphorus per hour per $10^{10}$ polymorphonuclear leukocytes, whereas the corresponding value for 75 healthy young women was 35 (table 1).

C. Elderly Subjects

Women at least 2 years postmenopausal and men aged 65 years or above, comprised this group, the mean enzyme value in males being 21 and the mean enzyme value in females 22 mg. of phosphorus per hour per $10^{10}$ polymorphonuclear leukocytes (table 1).

D. All Ages Combined

There was a total of 143 men and 160 women in the study. No significant differences were noted between leukocyte alkaline phosphatase in the two groups when all ages were combined ($p > 0.05$). The overall male mean was 27, that in the female group 31 (table 1). Variances in all groups were comparable (table 1). The overall distributions are compatible with Gaussian curves (figs. 1 and 2).

E. In Vivo Studies

I. Nineteen postmenopausal or surgically castrated women with carcinoma of the breast comprised this group: 10 were receiving no hormonal therapy, 6 were being treated with androgens and 3 with estrogens. The lowest leukocyte alkaline phosphatase values occurred in the androgen-treated group (table 3). No statistical comparisons were done because of the small size of the group.

II. Three normal women were studied during various phases of their menstrual cycle. The results are given in table 4, showing no essential change in this very small series.
Table 1.—Leukocyte Alkaline Phosphatase in Normal Healthy Subjects in Various Age Groups

<table>
<thead>
<tr>
<th></th>
<th>Number of Subjects</th>
<th>Mean Age (Years)</th>
<th>Age Range (Years)</th>
<th>Mean APA*</th>
<th>Log Mean APA</th>
<th>Standard Deviation S</th>
<th>Variance S²</th>
<th>Significance of Differences between Log Means</th>
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<td></td>
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<td></td>
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<tr>
<td>Children</td>
<td>Male</td>
<td>26</td>
<td>3 5/12</td>
<td>1/12-15</td>
<td>66</td>
<td>1.817</td>
<td>.196</td>
<td>.0380</td>
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<tr>
<td></td>
<td>Female</td>
<td>20</td>
<td>2 7/12</td>
<td>2/12-10</td>
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<td>.217</td>
<td>.0471</td>
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<td>Adults</td>
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<td>74</td>
<td>35</td>
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<td>.232</td>
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<td>Elderly</td>
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<td>72</td>
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<td>21</td>
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<td>.249</td>
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<td>Female</td>
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<td>65</td>
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<td>All subjects</td>
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<td>143</td>
<td>40</td>
<td>—</td>
<td>27</td>
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<td>.293</td>
<td>.086</td>
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<td></td>
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<td>42</td>
<td>—</td>
<td>31</td>
<td>1.497</td>
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*Alkaline phosphatase activity expressed as milligrams of phosphorus liberated per hour per 10¹⁰ polymorphonuclear leukocytes.

Table 2.—Children: Regression with Age

<table>
<thead>
<tr>
<th></th>
<th>Number of Subjects</th>
<th>Mean Age (months)</th>
<th>Log Mean Age</th>
<th>Log Mean APA*</th>
<th>Standard Deviation S</th>
<th>Regression Coefficient B</th>
<th>Significance: Fisher's t</th>
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<tr>
<td>Male</td>
<td>26</td>
<td>15.4</td>
<td>1.26</td>
<td>1.82</td>
<td>.195</td>
<td>-.175</td>
<td>.001 &lt; p &lt; .01</td>
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<tr>
<td>Female</td>
<td>29</td>
<td>18</td>
<td>1.22</td>
<td>1.95</td>
<td>.217</td>
<td>-.218</td>
<td>.001 &lt; p &lt; .01</td>
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</table>

*Alkaline phosphatase activity expressed as milligrams of phosphorus liberated per hour per 10¹⁰ polymorphonuclear leukocytes.

III. Pregnancy has been reported to be associated with elevated leukocyte alkaline phosphatase.7,14,15 In the present study, the results in 34 pregnant women are shown in table 3, where the mean leukocyte alkaline phosphatase value was 47 mg. of phosphorus per hour per 10¹⁰ polymorphonuclear leukocytes. This level is considerably higher (p < .01) than the average for non-pregnant women in the child-bearing age group.

F. In Vitro Studies

The effects of addition in vitro of 10 androgens and 8 estrogens on enzyme activity were tested. All hormones were tested on the leukocytes of 5 polycythemic subjects whose high enzyme values would lend themselves well to observations of enzyme activity inhibitions. The white cells of 2 patients (data of 1 patient shown in figure 5 a, b) demonstrated inhibition of alkaline phosphatase activity by androgens. No significant effect was found in the other 3 patients. There was no appreciable in vitro inhibition of any patient's leukocyte alkaline phosphatase by estrogenic hormones.

G. Saponization of Leukocytes

Leukocyte suspensions were treated with a variety of agents in attempts to increase the yield of enzyme activity. These treatments included freezing and thawing, various concentrations of sucrose,16 hand and electrical homogenization, ultraviolet light, ultrasonic disruption and various detergents. Most proved unsatisfactory to disrupt cells and granules. A marked increase in phosphatase activity was noted following saponin...
LEUKOCYTE ALKALINE PHOSPHATASE

Fig. 3.—Relationship between leukocyte alkaline phosphatase and age for girls. Solid line represents calculated regression line.

Fig. 4.—Relationship between leukocyte alkaline phosphatase and age for boys. Solid line represents calculated regression line.

treatment of leukocytes (table 5). Approximately a two-to-four fold increase occurred in 0.9 per cent saponin. The ratio between alkaline phosphatase activity of whole leukocytes and total saponin lysate was similar in all groups tested with no sex differences demonstrable. Pregnant women who had higher than normal whole leukocyte activities of this enzyme showed comparable increases following saponization of their leukocytes. Following high speed centrifugation, the postsaponization supernatant yielded phosphatase activities approximately one-half to two-thirds of the total saponin lysate activity ex-
Table 3.—Leukocyte Alkaline Phosphatase in Patients with Carcinoma of the Breast, Ambulatory and Clinically Well, All Either Post Menopausal or Surgically Castrated

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Subjects</th>
<th>Mean Age (Years)</th>
<th>Age Range (Years)</th>
<th>Mean APA*</th>
<th>Log Mean APA</th>
<th>S.D.</th>
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<td>None</td>
<td>10</td>
<td>62</td>
<td>48-72</td>
<td>48</td>
<td>1.682</td>
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<tr>
<td>Androgen</td>
<td>6</td>
<td>62</td>
<td>26-73</td>
<td>31</td>
<td>1.488</td>
<td>.158</td>
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<tr>
<td>Estrogen</td>
<td>3</td>
<td>70</td>
<td>60-78</td>
<td>60</td>
<td>1.783</td>
<td>.084</td>
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</table>

*Alkaline phosphatase activity expressed as milligrams of phosphorus liberated per hour per 10¹⁰ polymorphonuclear leukocytes.

Table 4.—Leukocyte Alkaline Phosphatase Activity during Menstruation in Three Normal Subjects

<table>
<thead>
<tr>
<th></th>
<th>Age (Years)</th>
<th>7 to 10 Days before Menses APA*</th>
<th>During Menses APA*</th>
<th>7 to 10 Days after Menses APA*</th>
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<td>T. L.</td>
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<td>L. H.</td>
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<td>41</td>
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<td>I. N.</td>
<td>47</td>
<td>90</td>
<td>82</td>
<td>75</td>
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*Alkaline phosphatase activity expressed as milligrams of phosphorus liberated per hour per 10¹⁰ polymorphonuclear leukocytes.

except in gravid women where the enzyme activity of the supernatant obtained after saponization was only one-fourth that of the total saponin lysate. Preliminary studies indicate that resaponization of the "nuclear" pellet releases significant amounts of additional enzyme activity.

DISCUSSION

Alkaline phosphatase activity of peripheral blood leukocytes at pH 9.3 is known to deviate from normal in certain disease states. It is high in polycythemia vera, some cases of myeloid metaplasia, infection, leukocytosis, leukemoid reactions, trauma, surgery and various "stressful" situations, such as myocardial infarction, diabetic acidosis and gout. It is also elevated in response to ACTH and adrenal steroids, in pregnancy and in mongolism. It is low in chronic myelogenous leukemia, paroxysmal nocturnal hemoglobinuria, hypophosphatasia and rarely in conditions such as aplastic anemia, infectious mononucleosis, idiopathic thrombocytopenic purpura and pernicious anemia in relapse. In none of the above reports is mention made of differences in this enzyme between men and women.

The fact that such differences exist, as described in this report, in the adult age group, requires explanation. Especially puzzling is the finding of equally high leukocyte alkaline phosphatase levels in male and female children and the extremely high values of this enzyme in newborns (reported by Scorza and associates). It seems reasonable to assume that endocrine factors play a role in the regulation of this enzyme. Previous investigations were directed toward studying the effects of androgens and estrogens on renal, liver and intestinal alkaline phosphatases in animals. In mice, there is a decrease of renal alkaline phosphatase following the administration of various androgens, but no change of liver or intestinal alkaline phosphatase oc-
Fig. 5.—(A) Inhibition of leukocyte alkaline phosphatase activity by androgens and failure of inhibition by estrogens. White cells from L. C polycythemia vera. (B) Inhibition of leukocyte alkaline phosphatase activity by androgens and failure of inhibition by estrogens. White cells from L. C polycythemia vera.

curs. Estrogens had no effect on kidney or liver alkaline phosphatases when given to mice. In rats, androgens produce a slight increase in kidney and liver alkaline phosphatase, castration produces a decrease in the kidney alkaline phosphatase which is restored toward normal by various
Table 5.—Increase in Leukocyte Alkaline Phosphatase Activity Following Saponization of Whole Leukocytes

<table>
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<th>Number of Cases</th>
<th>Mean Age (Years)</th>
<th>Age Range (Years)</th>
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<th>Log Mean APA</th>
<th>S.D.</th>
<th>Mean APA</th>
<th>Log Mean APA</th>
<th>S.D.</th>
<th>Mean APA</th>
<th>Log Mean APA</th>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>34</td>
<td>19–60</td>
<td>17</td>
<td>1.242</td>
<td>.245</td>
<td>58</td>
<td>1.763</td>
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<td>.283</td>
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<td>33</td>
<td>19–48</td>
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<td>.247</td>
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<td>.167</td>
<td>40</td>
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<tr>
<td>Male</td>
<td>8</td>
<td>73</td>
<td>65–82</td>
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<td>.212</td>
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<td>69</td>
<td>54–83</td>
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<td>64</td>
<td>1.806</td>
<td>.245</td>
<td>41</td>
<td>1.617</td>
<td>.245</td>
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<td>Pregnant subjects</td>
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<tr>
<td></td>
<td>34</td>
<td>26</td>
<td>17–45</td>
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<td>1.673</td>
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<td>126</td>
<td>2.098</td>
<td>.185</td>
<td>30</td>
<td>1.482</td>
<td>.218</td>
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*Alkaline phosphatase activity expressed as milligrams of phosphorus liberated per hour per 10^10 polymorphonuclear leukocytes.
androgenic steroids. Investigation of rabbit kidney and liver alkaline phosphatase showed no inhibition of these enzyme activities by estradiol and estrone but strong inhibition by some of their phosphorylated derivatives, notably, estrone-3-phosphate and estradiol-3-17-diphosphate. These phosphorylated estrogens were not hydrolyzed by the phosphatase.

Thyroidectomy produces a decrease in kidney alkaline phosphatase which is restored to above normal by therapy with testosterone, whereas liver alkaline phosphatase increases after thyroidectomy and is unaltered by testosterone. Adrenaline inhibits serum, bone, intestinal mucosa and milk alkaline phosphatases. Parathyroid extracts produce no effect on male rat kidney alkaline phosphatase.

From these confusing and somewhat contradictory reports on various tissue alkaline phosphatases in animals two facts emerge: androgens inhibit renal alkaline phosphatase in mice and certain estrogens inhibit renal alkaline phosphatase in rabbits. The present study indicates that androgens inhibit human leukocyte alkaline phosphatase. Low values in men as compared to women and as compared to children support this thesis, as do the fragmentary results in hormone-treated breast-cancer patients and in test-tube experiments with hormones.

Other influences on leukocyte alkaline phosphatase activity, probably also hormonal, are clearly present. Girls as well as boys show gradually decreasing activity with advancing age towards puberty, and women have levels which are distinctly lower than those of children. Levels of this enzyme activity in women go up with pregnancy, down with the menopause, but (in these experiments) have not been shown to vary during the menstrual cycle. In the test tube, none of the estrogens tested had any consistent effect on leukocyte alkaline phosphatase. The specific phosphorylated estrogens, estrone-3-phosphate and estradiol-3-17-diphosphate, shown to inhibit rabbit kidney and liver alkaline phosphatase, were tested but showed no effect on this enzyme in human leukocytes. How the female sex hormones influence leukocyte alkaline phosphatase is not clear. Perhaps estrogens and progestins have mutually antagonistic effects.

Very high values of this enzyme in young children and gradually decreasing levels toward puberty suggest a possible stimulating role for growth hormone.

An alternative explanation for the observed differences in leukocyte alkaline phosphatase between men and women is that genetic determination plays a major role. This seems unlikely; the absence of differences between boys and girls and old men and women suggests that leukocyte alkaline phosphatase activity is a secondary sex characteristic.

Leukocyte alkaline phosphatase activity, as measured in the present and other studies, depends on release of this enzyme from granules of leukocytes during the period of incubation. The level of enzyme activity detected must depend on two factors: the content of enzyme per $10^{10}$ cells, and the rate of rupture of granules. Enzyme content may be genetically determined, and may also, in an individual cell, depend on the age of the cell; that is, it is...
likely that protein synthesis is at a minimum in mature neutrophilic leukocytes, and therefore, that cells which have been circulating longest have, because of attrition of age, the lowest enzyme content. Enzyme release from granules may be influenced by changes in the intracellular environment.

Might the sex differences be explained by differences in release of enzyme? Saponin treatment of leukocytes was found to increase the yield of this enzyme two-to-four fold and it was hoped that such treatment would eliminate the factor of rate of release of enzyme from consideration, and so permit measurement of total enzyme content. However, saponization led to proportionally similar enhancement of the enzyme activity in all subjects studied. But since retreatment with saponin of the “nuclear” pellet released significant additional activity, no inferences can be drawn about total enzyme content. Release of more enzyme activity by saponin might simply mean more easily ruptured granules. Until it is possible to release all the activity from all the granules of human leukocytes, it will not be known to what extent enzyme content and enzyme release contribute to conventional determination of alkaline phosphatase in human leukocytes.

**Summary**

Leukocyte alkaline phosphatase activity has been noted to be different in men and women. The mean leukocyte alkaline phosphatase activity for 74 normal men, aged 19 to 60 years, was 23 mg. of phosphorus per $10^{10}$ polymorphonuclear leukocytes per hour. The corresponding mean value for 75 normal young women, age 19-48 years, was 35 ($p < .001$). No significant differences between boys and girls occurred until the time of puberty. After the menopause, the values for women approached the values for men. Women treated with androgens had lower leukocyte alkaline phosphatase activity than did control women. These results suggest that androgenic hormones inhibit this enzyme, and that other, as yet undefined endocrine influences, also affect its level of activity.

In vitro tests with various concentrations of androgens and estrogens failed to provide conclusive evidence of direct effect on leukocytes although some degree of direct inhibition by androgens was suggested. Studies using saponin to effect enzyme release from leukocyte granules did not demonstrate whether the differences between men and women are differences of enzyme release or of content of leukocyte alkaline phosphatase.

**Summario in Interlingua**

Ha essite notate que le activitate de leucocytic phosphatase alcalin differe inter masculos e feminas. Le valor medie del activitate de leucocytic phosphatase alcalin in 74 masculos normal de etates de inter 19 e 60 annos esseva 23 mg de phosphoro per $10^{10}$ leucocytos polymorphonucleari per hora. Le correspondente valor medie in 75 normal juvene femininas de etates de inter 19 e 48 esseva 35 ($p < 0,001$). Nulle significative differentia esseva inter pueros e pueras usque al tempore del pubertate. Post le menopause, le valores pro feminas se approximava a illos pro masculos. Feminas tractate con
LEUKOCYTE ALKALINE PHOSPHATASE

androgenos habeva plus basse leucocytic activitates de phosphatase alcalin que feminas de controlo. Iste resultatos pare indicar que hormones androgene inhibi le enzyme in question e que altere (non ancora definite) influentias endocrin etiam affice le nivello de su activitate.

Tests in vitro con varie concentrationes de androgenos e estrogenos provideva nulle evidentia conclusive de un directe effecto super le leucocytos. Del altere latere, un certe grado de inhibition directe per androgenos se suggerева. Studios in que saponia esseva usate pro effectuar le liberation de enzyme ab le granulos leucocytic non succedeva in demonstrar si le differentias inter masculos e femininas es differentias in le liberation del enzyme o in le contento leucocytic de phosphatase alcalin.

REFERENCES


LEUKOCYTE ALKALINE PHOSPHATASE

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