act with lipopolysaccharides, thus interfering with their attachment to erythrocytes(11-15).

The present investigation and our previous report(1) suggest that lipopolysaccharides (endotoxins) and their lipoid A component render a certain antigen non-immunogenic; these mixtures, nevertheless, react well with CA antibodies *in vitro*. Thus, these findings are the reverse of those of haptens, for the latter become immunogenic upon attachment to certain foreign proteins. Further investigation to determine the precise mechanism of this inhibitory effect on immunogenicity of lipopolysaccharides and their lipoid A component may yield information on general aspects of the antibody response.

Summary. Five preparations of lipoid A, obtained from highly purified lipopolysaccharides (endotoxins), were shown to inhibit the antibody response to the common antigen (CA) of enteric bacteria. This inhibition occurred when mixtures of antigen and lipoid A (100 μg per injection) were prepared in ethanol *in vitro* and then redissolved in water for immunization. Only one of these 5 preparations was markedly effective as inhibitor when mixtures of lipoid A and antigen were prepared *in vitro* in aqueous solution. Quantitation of one of the preparations showed that as little as 20 μg per injection of lipoid A decreased, but did not abolish, the antibody response. Simultaneous injection of antigen and lipoid A into different veins failed to interfere with CA antibody formation. Lipoid A also interferes with the antibody response, when mixtures prepared in ethanol were injected into the footpad with Freund's adjuvant or in the presence of autologous erythrocytes. It is concluded that lipoid A interacts with CA and that antibody formation is suppressed as a result of this interaction.

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Received July 15, 1965. P.S.E.B.M., 1965, v120.

Thymidylate Dephosphorylation in Granulocytes.* (30541)

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Normal granulocytes of human beings and most other mammals contain an alkaline phosphatase (AP) capable of hydrolyzing a variety of natural and synthetic phosphate monoesters. This enzyme has been extensively studied by other workers both biochemically using leukocyte homogenates and cytochemically in blood smears. Most of

* This investigation was supported by USPHS Research Grant CA08063-01.

these previous studies have utilized synthetic AP substrates such as aryl phosphates or sodium beta glycerophosphate (BGP). The actual physiologic substrates and metabolic functions of granulocyte AP have remained unknown. We have recently suggested that granulocyte AP may utilize thymidylic acid (TDRP) as a natural substrate and by this means regulate DNA synthesis(1). In the present study, human leukocytes were tested biochemically and cytochemically for AP activity with a variety of synthetic and natural substrates. The present results demonstrate that TDRP as well as other naturally occurring nucleotides can readily be dephosphorylated in intact granulocytes.

Materials and methods. Alkaline phosphatase activity was assessed in blood and bone marrow of patients selected to present the full range of values as predicted from the literature(2,3,4,5,6,7). Smears for cytochemical studies were prepared from blood obtained by finger puncture or from aspirated bone marrow. The cell smears were promptly fixed by immersion for 45 seconds in 10% formalin in 95% methanol at —10°C. The fixed slides could then be stored at —20°C for at least 2 weeks without decrease in enzyme activity. These slides were processed for AP using either an acid-salt technique modified from that of Gomori(8) or the azo-dye method of Kaplow(2).

In the modified acid-salt method the fixed slides were incubated for 30 minutes at 37°C in a solution of 1% $CaCl_2$ containing the substrate at 0.002 M with 0.0013 M $MgCl_2$ adjusted to pH 9.0 with tris buffer. The slides were then washed briefly in water, immersed 5 minutes in 2% cobalt acetate, washed again, and dipped 5 minutes in 1.5% ammonium sulfide. The completed slides were then counterstained with Wright's stain. Kaplow's cytochemical method(2) for granulocyte AP was followed using sodium alpha naphthyl acid phosphate, fast blue RR, and Mayer's aqueous hematoxylin as counterstain.

All stained slides with either method were coded and randomized prior to examination. Assessment of cytochemical granulocyte AP activity was carried out by the scoring method of Kaplow(2).

Biochemical assay of buffy coat homogenates for AP activity upon selected monoesters was done as described by Valentine and Beck(7) and results expressed as mg P/hr/10^5 leukocytes. The following substrates were evaluated: Sodium beta glycerophosphate (BGP), thymidylic acid (TDRP), deoxyadenosine-5-monophosphate (5-ADRP), cytidine-2,3-cyclic monophosphate (2,3-CRP), deoxycytidine-5-monophosphate (5-CDRP), deoxyuridine-5-monophosphate (5-UDRP), deoxyguanosine-5-monophosphate (5-GDRP), adenosine-5-monophosphate (5-ARP) and adenosine-2,3-cyclic monophosphate (2,3-ARP).†

Results. Cytochemical granulocyte AP activity for several substrates was evaluated with the acid-salt method in smears from 6 patients. The results are shown in Fig. 1. Dephosphorylation of all substrates appeared to vary with the type of patient tested. The efficacy of TDRP and other naturally occurring nucleotides as substrates for granulocyte AP is apparent. In all blood smear and bone marrow slides evaluated for cytochemical AP activity, this enzyme was detected only in neutrophilic bands and polymorphonuclear granulocytes. No activity for any substrate was seen in other cell types, including various types of leukemic cells. Smears from rabbits, guinea pigs and horses showed similar properties, but regularly yielded scores in excess of 200 for all substrates.

Granulocyte AP activity was consistently high in non-leukemic leukocytosis, as expected. Of interest, however, were high thymidylate, B-GP and azo-dye AP scores in the rare circulating granulocytes seen in smears from 4 cases of transient leukopenia in patients receiving cyclophosphamide, 5-fluorouracil, or chlorpromazine. With subsequent rise of the white cell counts towards normal, the granulocyte AP scores decreased proportionately. A typical case is depicted in Fig. 2.

Biochemical assay of buffy coat AP activ-

† Sodium Beta-glycerophosphate supplied by Matheson, Coleman & Bell, East Rutherford, N. J. Adenosine-5-monophosphate supplied by Schwarz BioResearch, Mt. Vernon, N. Y. All other nucleotides supplied by California Corporation for Biochemial Research, Los Angeles.

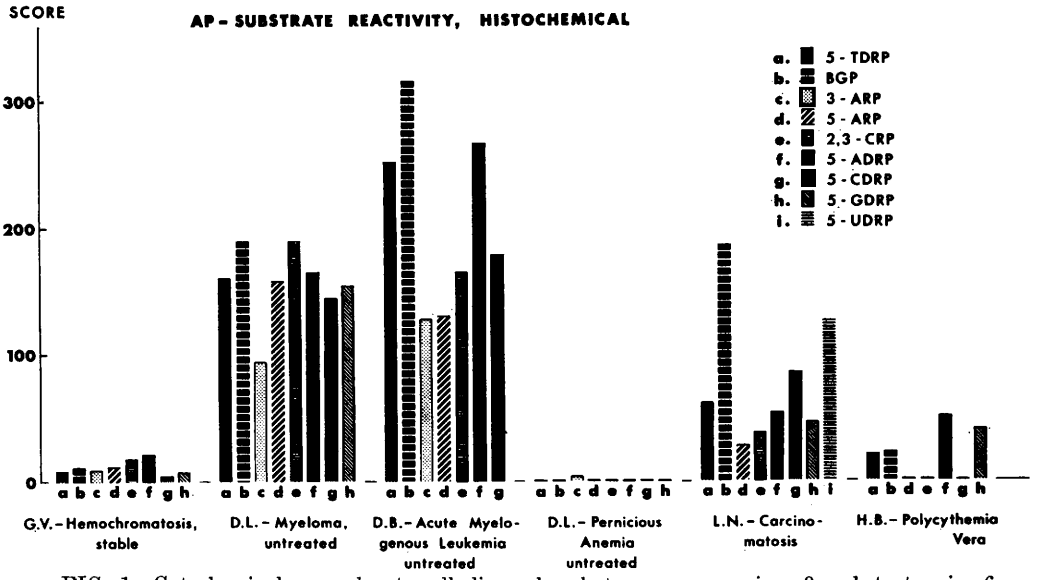


FIG. 1. Cytochemical granulocyte alkaline phosphatase scores using 9 substrates in 6 patients.

ity with TDRP *vs* B-GP was done in 26 selected patients. These results are shown in Fig. 3. Here, TDRP showed a greater reactivity than B-GP of, on the average, 1.6:1. A similar study using cytochemical instead of biochemical technique, in 35 patients, indicated roughly 1:1 correlation (Fig. 4).

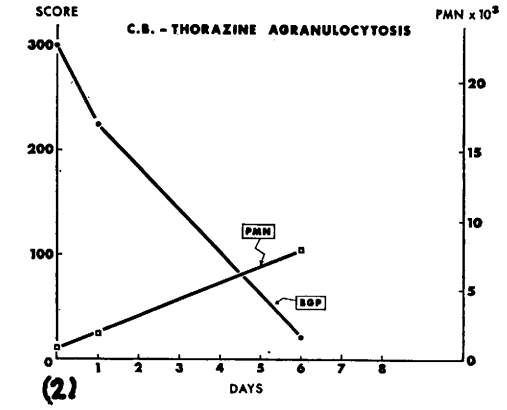
Comparison of acid-salt and azo-dye scores also demonstrated linear correlation, although the azo-dye technique appeared somewhat more sensitive.

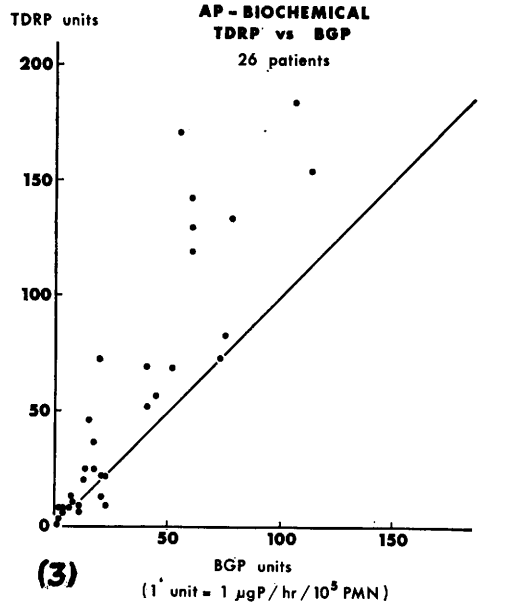
Discussion. The presence in circulating granulocytes of an alkaline phosphomonoesterase was first described in 1931(9). This enzyme is generally found only in neutrophils although it is said to occur in the eosinophils of certain species(10). Enzyme activity is normally high in granulocytes of the hamster, burro, steer, guinea pig, rabbit and rat, and low in those of the mouse, cat and dog, with man occupying a low-intermediate position(11).

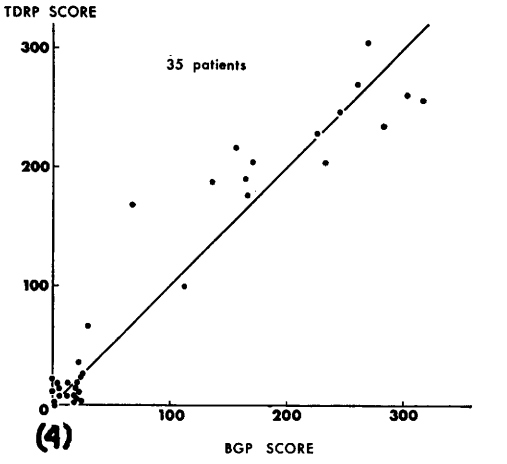
The considerable variation of human granulocyte AP activity in health and disease has been studied by many investigators, using B-GP and aryl phosphates. In a recent representative large series, totaling 882 determinations, Martinez-Maldonado *et al* (5) found elevated scores (over 70) in non-leukemic leukocytoses, Hodgkin's disease, polycythemia rubra vera, most cases of chronic lymphocytic leukemia, and sporadically in a variety of other diseases. Low values (less than 5) were routinely found in chronic myelogenous leukemia, and occasionally (35 instances) in a variety of other conditions, hematologic or otherwise, active or quiescent. Our experience has been similar.

Generally AP activity is not uniformly distributed among granulocytes. Our cytochemical results with 9 substrates tested demonstrated variability of activity among granulocytes, and dephosphorylation was not seen in other cells. Enzyme-laden cells seemed to show a nearly equal avidity for the several organophosphates used. These results suggest the action of but one enzyme in dephosphorylating all the substrates examined.

Most previous workers have tested granulocyte AP with azo dyes or B-GP, substances not normally present *in vivo*. Our results show that granulocytes can act on physiologic substrates such as TDRP and other important nucleotides. We have been interested in TDRP and other nucleotides as substrates for AP of granulocytes and other tissues(1). The TDRP required for DNA synthesis can be supplied in adequate quantities by methylation of uridylic acid through the action of







thymidylate synthetase. The only alternate pathway, TDR phosphorylation by thymidine kinase, is probably of low capacity. Hence, DNA synthesis might reasonably be presumed susceptible to inhibition by any agent that degrades TDRP. Trubowitz(12) tested an AP preparation purified from human granulocytes for its biochemical activity upon 32 substrates. His results showed that TDRP was the most active of all substrates tested. Elsewhere, we have demonstrated that other tissue AP preparations can also readily hydrolyze TDRP(1). Our present cytochemical and biochemical results in demonstrating that granulocyte AP can readily dephosphorylate TDRP, support the functional role proposed above.

One important problem regarding a physiological role of AP enzymes has been the very unphysiological alkaline pH optima presumably required for their activity. Yet, Ross *et al*(13) have pointed out that AP pH optima are strikingly dependent upon substrate concentration. When substrate concentrations were reduced to very low levels, the pH optimum shifted to near neutral. This may be very relevant to TDRP dephosphorylation in view of the low levels of TDRP present in most cells.

Summary. Human blood and marrow leukocytes were studied both cytochemically and biochemically for alkaline phosphatase activity. Naturally occurring nucleotides, as well as synthetic compounds, were tested as substrates for leukocytes from varied patients. All substrates reacted similarly with granulocytes from normal subjects. Granulocytes from patients with selected diseases showed proportional changes in reactivity for all substrates. These results suggest the presence of one common alkaline phosphatase. Dephosphorylation of thymidylate was consistently demonstrable. This unique deoxynucleotide

FIG. 2. Serial granulocyte alkaline phosphatase scores using sodium beta-glycerophosphate, and granulocyte (PMN) counts in a patient recovering from chlorpromazine induced agranulocytosis.

FIG. 3. Biochemical comparison of thymic acid and sodium beta-glycerophosphate dephosphorylation in granulocytes of 26 patients.

FIG. 4. Cytochemical scores, thymidylic and (TDRP) *vs* sodium beta-glycerophosphate (BGP).

may be an important natural substrate for granulocyte alkaline phosphatase.

The authors wish to express their appreciation to Miss Hilda Boyer and Miss Sylvia Brickson for technical assistance.

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Received July 16, 1965. P.S.E.B.M., 1965, v120.

In vitro Uptake of Isotopically Labeled RNA by Mammalian Spleen Cells.* (30542)

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The incorporation of nucleic acid or nucleoprotein into mammalian cells has been reported by several investigators(1-3). Bensch and King(4) noted the uptake of particles containing a coacervate of high molecular weight mouse Sarcoma DNA and protein, by a strain of Earle's L cells *in vitro*. Higginbotham(5) reported that fibroblasts incorporate DNA by phagocytosis *in vivo*. Kay (3) presented evidence for incorporation of 2 to 4% of the administered DNA into Ehrlich-Lettre ascites cells *in vitro*. It was found that the DNA retained its macromolecular structure in the host cell. Borenfreund *et al* (6) reported that tritium labeled RNA from pneumococci and human leucocytes was incorporated by growing Hela cells. The amount of DNA incorporated was equivalent to 10% of the normal Hela cell complement. Gartler(7) described uptake of DNA by Earle's L cells of 0.5 to 3% of the DNA administered. A smaller amount was reportedly degraded upon uptake, and the fragments incorporated into host DNA. Schwarz and Rieke(8) demonstrated *in vivo* and *in vitro* incorporation of tritium labeled RNA from normal mouse liver into isologous mouse ascites tumor cells by the method of autoradiography. The cells reported as incorporating RNA were large and medium lymphocytes. Amos and Moore(9) have shown that RNA from various sources stimulates the synthesis of protein in chick embryo fibroblasts. However, the magnitude of the response was not correlated with the concentration of exogenous RNA. This disproportion was apparently due to the rapid depolymerization of the RNA to acid soluble products by ribonucleases of the media. Amos and Kearns (10) have demonstrated significant uptake of P^{32} labeled RNA by chick embryo fibroblasts, and have suggested that the degradation RNA can be prevented by various additives.

In view of the reported ability of RNA extracted from the spleens of immune animals, to transfer the capacity for antibody synthesis to nonimmune spleen cells(11,12), it seemed desirable to examine the conditions necessary to obtain measurable cellular uptake of rat spleen RNA by isologous spleen

* Supported by research grant AM 01024 from Nat. Inst. Health, and through Marquette Univ. Committee on Growth and Cancer.