dimethyl groups). It furnished a monoacetate, $C_{32}H_{48}O_4$, m.p. 300–305°, $[\alpha]_D^{32}$ — 26.4° ($CHCl_3$). Fraction E on hydrolysis with alcoholic caustic potash furnished machaerinic acid[4]. Formation of machaerinic acid suggests fraction E to be 28 → 21 lactone of 3β:21β-dihydroxy-olean-12-ene-28-oic acid with conformational alternation[7,8] in ring D or E, since the steric requirement for 28 → 21 lactonization is not fulfilled in *cis*-locked D/E rings with all chair conformation.

Fraction F, $C_{32}H_{52}O_4$, m.p. 198–199°, $[\alpha]_D^{31}$ + 81° ($CHCl_3$), gave a diacetate, $C_{36}H_{56}O_6$, m.p. 223–224°, $[\alpha]_D^{32}$ + 85.45° ($CHCl_3$). The IR-spectrum showed bands at 3400 cm^{-1} (hydroxyl), 1710 cm^{-1} (ester carbonyl), and at 1365 and 1370 cm^{-1} (*gem*-dimethyl). Fraction F responded to the characteristic test of a triterpene in the Liebermann-Burchard reaction and in tetranitromethane, and was identified as ethyl ester of machaerinic acid. This acid, on treatment with diazoethane, gave Fraction F. The latter was proved to be formed during isolation.

Fraction G, $C_{30}H_{46}O_4$, and Fraction H, $C_{30}H_{48}O_4$, were found to be identical with proceragenin A and proceragenin B respectively obtained from the acid sapogenin fraction. Proceragenin A and proceragenin B on hydrolysis with alcoholic caustic potash yielded acids, which on treatment with diazomethane or on standing with alcoholic hydrochloric acid gave back proceragenin A and proceragenin B respectively. This is perhaps the reason why these compounds are encountered from both the acid and neutral sapogenin fractions during isolation.

Further work on the constitution of proceragenin A and proceragenin B is under progress.

Zusammenfassung. Aus dem Samen von *Albizzia procera* Benth wurden ausser Machaerinsäure 4 triterpenoide Sapogenine isoliert (Verbindung A und B, Proceragenin A und B). Es wurde bewiesen, dass die Verbindung A das 28 → 21-Lacton der Machaerinsäure mit einer Konformationsänderung am Ring D oder E ist, während die Verbindung B als Äthylmachaerinat identifiziert wurde. Bei Proceragenin A und B handelt es sich um Dihydroylactone.

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The Indigogenic Reaction for Histochemical Demonstration of Alkaline and Acid Phosphatase

Previous work in this laboratory described the application of the indigogenic principle to the histochemical demonstration of leucine aminopeptidase[1], β-glucosidase[2], β-galactosidase[3] and N-acetyl-β-glucosaminidase[4]. In the present study we have extended this principle to specific localization of alkaline and acid phosphatase, utilizing 5-bromo-4-chloro-3-indolyl phosphate (I) and 5-bromo-6-chloro-3-indolyl phosphate (II) (as the para-toluidine salts) as substrates. Both products were synthesized according to methods described recently by HORWITZ and co-workers[5,6]. The substrates offer the advantage of a precise enzyme localization with no or very slight diffusion. Moreover, the substrates afford a simple and direct method for demonstration of hydrolytic enzymes without the need for a coupling reaction. Compound I yields a blue-green deposition of indigo whereas compound II gives a magenta colored precipitate.

Methods. Tissues from mouse, rat, guinea-pig and man were used for this study. Representative pieces of tissue from each organ were removed and cut into blocks 2–4 mm in thickness, and quick-frozen by placing the tissue in a glass tube and immersing it in a Dewar flask containing acetone and dry ice at — 70 °C. The tissues were embedded in O.C.T. (optimal cutting temperature) compound, purchased from Lab-Tek, composed of water-soluble glycols and resins matched to a specific cutting zone temperature of — 20 °C to — 35 °C. The embedded tissue was then placed on the quick-freeze bar of a Lab-Tek cryostat for 1 min until the embedding medium was frozen and became the proper consistency for cutting 6 μ sections at — 20 °C. After cutting, the sections were attached to warm slides and fixed for 5 min in cold acetone, passed through 95% and 85% acetone and then rinsed in distilled water. All solutions were maintained at 4 °C for preservation of enzymatic activity. The slides were then air dried to prevent the formation of ice crystals and stored at — 25 °C until incubated in the specific substrate solution. Fresh frozen and cold acetone-fixed sections were incubated for 2 h in solutions containing para-toluidinium 5-bromo-4-chloro-3-indolyl phosphate or para-toluidinium 5-bromo-6-chloro-3-indolyl phosphate, depending on the desired color of the end-product. The solutions were made specific for acid and alkaline phosphatase by varying the pH of the buffer used for incubation. The incubating solution employed for alkaline phosphatase is as follows: 14.0 ml *Tris* buffer pH 8.4–9.3, 0.05 *M*; 1.0 ml para-toluidinium 5-bromo-4-chloro-3-indolyl phosphate or para-toluidinium 5-bromo-6-chloro-3-indolyl-phosphate (2.1 mg/ml of dimethylformamide), 0.00031 *M* (final concentration); 1.0 ml $MgCl_2$ 0.005 *M*

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(final concentration); and 4.0 mg spermadine trihydrochloride 0.001 *M* (final concentration). The total volume in the Coplin jar is 16.0 ml. The incubating solution utilized for the acid phosphatase had the following variations: 14.0 ml acetate buffer, pH 5.0, 0.1 *M*; 1.0 ml substrate (as listed above); 1.0 ml $MnCl_2$, 0.000001 *M* (final concentration); and 4.0 mg spermadine trihydrochloride, 0.001 *M* (final concentration), for a total volume of 16.0 ml. After incubation the slides were washed briefly in tap water and mounted in glycerol-gel for microscopic examination.

Results. Tissues from all the major organs of mouse, rat, guinea-pig, and man were examined for alkaline and acid phosphatase activity. Substrate I gave a final blue-green indigo at the site of enzyme localization, while substrate II yielded a magenta precipitate. The best results were obtained with substrate I, and the description of the experimental results is based on the sections stained utilizing substrate I. Substances which accelerated the histochemical reaction were Mg, Mn and Zn, whereas inhibition of the reaction occurred with EDTA and $KMNO_4$. Mn accelerated the histochemical reaction for acid phosphatase, while fluoride and oxalate inhibited the reaction.

Our histochemical observations indicated that alkaline phosphatase was generally of a high order of activity in normal mouse, rat, guinea-pig, and human tissues. The organs showing the greatest alkaline phosphatase activity were proximal and distal convoluted tubules of the kidney, epithelium of stomach, small intestine and large intestine, epididymis, adrenal cortex, salivary gland, uterus, vascular endothelium generalized, and peripheral blood neutrophils. In the stomach, small and large intestine, the reticular cells of the lamina propria and vascular endothelium also gave a strong alkaline phosphatase reaction. Alkaline phosphatase activity was present in the vascular endothelium of renal vessels and glomeruli. Organs showing lesser activity were pancreas, thyroid, ovary, oviducts, testis, preputial gland, liver, reticulum cells in lymph node, spleen and gastrointestinal tract (Figure 1).

The organs showing the greatest acid phosphatase activity were glandular epithelium of prostate, epididymis, and seminal vesicle, proximal and distal convoluted tubules of the kidney, preputial gland, uterine endometrium, epithelium of the cervix, glandular epithelium of salivary glands, and adrenal cortex. Lesser activity was observed in reticular cells in lymph nodes, spleen and gastrointestinal tract, in liver, pancreas, epidermis and cerebellum (Figure 2).

Discussion. This paper describes the histochemical demonstration of alkaline and acid phosphatase utilizing 2 new indolyl chromogens. We obtained precise enzyme localization with little or no diffusion.

Very little work has been reported utilizing indoxyl phosphate as a histochemical substrate. SELIGMAN et al.[7] first reported the use of indoxyl phosphate (sodium salt) for the histochemical demonstration of alkaline phosphatase. However, the substrate yielded a macrocrystalline product which rendered it unsuitable. Subsequently, HOLT[8] described the application of calcium 5-bromo-indoxyl phosphate to the localization of the same enzyme in which an excellent microcrystalline end-product was demonstrated. Unfortunately, the synthesis of the substrate was never described.

PEARSE[9] has indicated the need for further work in the application of the indigogenic principle to the localization of phosphatase activity. Failure to expand this area to date is in all probability due to the difficulty in the requisite substrate-synthesis. Consequently, the demonstration of phosphatase activity customarily depends upon the azo-dye methods and the procedure of GOMORI[10]. Much work has been performed in the histochemistry of phosphatase since the GOMORI[10] and TAKAMATSU[11] methods were introduced. They include simultaneous azo-dye methods (unsubstituted naphthols) of MENTEN, JUNGE and GREEN[12]; simultaneous azo-dye methods (substituted naphthols) of RUTENBURG[13]; and non-coupling azo-dye methods of LOVELESS and DANIELLI[14]. These methods involve coupling or azo-dye techniques.

Recently TSOU and SU[15] prepared an unsubstituted indoxyl phosphate and used it for a colormetric method

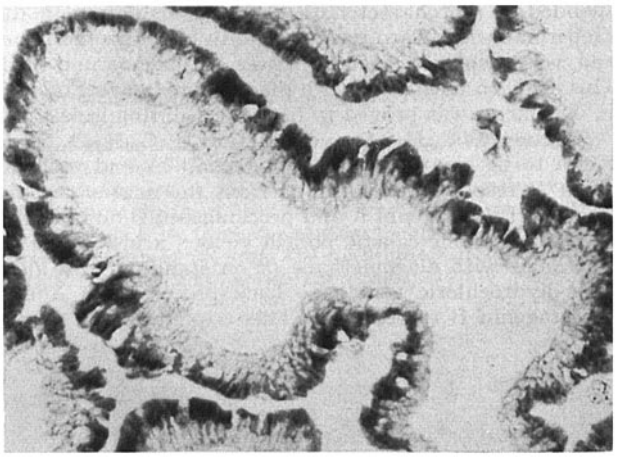

Fig. 1. Section of human small intestine demonstrating the marked alkaline phosphatase activity in the epithelial cells. × 235.

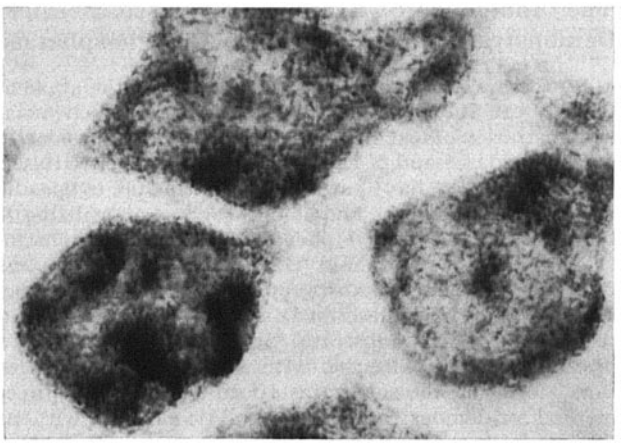

Fig. 2. Section of human kidney demonstrating granular, lysosomal acid phosphatase activity of renal tubular cells. × 1100.

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for alkaline phosphatase. They noted that free formaldehyde, when present, inhibited the oxidation, but alkaline phosphatase in formalin-fixed tissues remained active for this substrate. It would appear that this type of substrate may soon be applicable for determination of alkaline and acid phosphatase of serum in the clinical laboratory.

Alkaline phosphatase is generally regarded as a microsomal enzyme and the optimum pH varies between 8.5 and 10.0. Our histochemical reactions correlated with previous reports concerning optimum pH and distribution and activity in homogenate data[16-18].

In regard to acid phosphatase, granules and droplet formation occurred at the site of the enzyme activity which is generally considered to be lysosomal. Cold acetone fixation was found to be the best method for tissue preparation for histochemical demonstration of the enzymes[19].

Résumé. Le principe indigogénique à la détection histochimique des phosphates alcalins et acides a été appliqué. Ces substrates ont l'avantage de permettre une localisation précise de l'enzyme, sans ou avec minime diffusion. C'est aussi une méthode simple et directe pour la mise en évidence de ces enzymes sans passer par une réaction couplée. Un bisindigo très insoluble a lieu apparaît aux points d'activité enzymatique. Le 5-bromo-4-chloro-composé donne un indigo bleu-vert tandis que le 5-bromo-6-chloro-composé donne un indigo magenta.

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Ribonuclease Activity in Leucocytes of Hyperimmunized Rabbits

Ribonuclease has been described in rabbit polymorphonuclear leucocytes[1,2] and the enzyme is associated with specific granules. These specific granules have been described by Cohn and Hirsch[3] as lysosomal in nature. Following a cytochemical technique described by Atwal et al.[4], while studying the enzymatic properties of the specific granules of PMN leucocytes of Swiss mice, a semiquantitative survey was attempted on ribonuclease positive granules of rabbit PMN leucocytes in which earlier biochemical investigations indicated a high concentration of ribonuclease.

2 rabbits (1 of the animals was inoculated with an attenuated strain of *Coxiella burneti* for hyperimmune Q serum) were bled from the ear vein. The air-dried coverslip preparations were fixed in cold formalin vapors, and ribonuclease activity was measured cytochemically as described by Atwal et al.[4]. The leucocytes of these animals showed a distinct cytochemical dichotomy by showing a difference in the number of positive reactive granules. The vaccinated rabbit showed a higher number of PMN leucocytes, and every cell had more positive granules than the leucocytes of the normal rabbit (Figure 1). The positive granules were distributed throughout the whole extent of the cytoplasm, while in normal rabbits these granules were limited to the perinuclear zone of the cytoplasm (Figure 2). The routine hematological data indicated a distinct leucocytic reaction in the vaccinated rabbit. The cytoenzymatic dimorphism, although shown in 2 animals, was consistent in 3 successive experiments. This observation prompted a further study of enzymatic concentration to see if this phenomenon of increased ribonuclease activity had a correlation with the course of immunological response of the vaccinated animals. The cytochemical behavior of 5-nucleotidase activity in rabbit leucocytes during experimental infection with mixtures and single strains of various staphylococci etc., has been described by Szmigielski et al.[5]. They observed significant changes in 5-nucleotidase activity of infected animals in polymorphonuclear leucocytes in peripheral blood. During the present study a similar situation occurred when maximum enzyme activity was observed on the tenth day following the first inoculation. This coincided with the first peak phase in the antibody titer. Then followed a decline in the enzyme activity in PMN leucocytes. It again reached its previous high concentration level on the 28th day. This second rise in enzyme activity coincided with the second peak phase of the antibody concentration in the plasma[6]. It is speculated that the function of intracellular ribonuclease is to degrade the unwanted RNA[7]. Rabbit leucocytes have been described as having a rapid turnover in unstable RNA[8].

The question is: Why would the leucocytes show increased ribonuclease when the animal is in the process of active antibody formation? Are the circulating PMN leucocytes actively functional as phagocytes? The increased lysosomal ribonuclease indicated that this might be the case. The digestive enzymes of lysosomes and their role in the formation of phagosomes for the engulfed

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