peated bleeding is necessary because of traumatic effects and the technical difficulty of obtaining repeated blood samples through this route. Bleeding from small vessels such as those of the ear lobe is difficult and not satisfactory when several blood samples are required within a short period of time.

The technique described here has been found simple and reliable in accomplishing this work. The procedure used in this laboratory allows us to draw easily small amounts of blood up to 14 times from the same guinea pig in one day. Although our experience has been limited, we believe the procedure may be used on rats as well. It has not been tried on other species.

Equipment

1 250-w, infrared ray lamp.

3% Sodium citrate solution.

Microscope slides with one or two concavities. The slide and capillary pipettes may be dry or may have been moistened with the sodium citrate solution with further drying.

Capillary tip pipettes.

Curved-on-flat dissecting scissors, 115 mm long.

Small metal spatula.

The animal's foot is cleaned to remove all interfering dirt, rinsed with the sodium citrate solution, and thoroughly dried with cotton or gauze. The nail is cut just at its insertion, giving the scissors an inclined position (Fig. 1). The foot is placed about 15 cm from the infrared ray lamp for 10 sec, which is enough to provoke dilatation of the vessels and to facilitate hemorrhage. Well-fed animals can be bled even without this irradiation. In certain infections or other pathological states, however, this detail is important, since sometimes spontaneous hemorrhage cannot be obtained.

Two bleedings can be made from the same nail insertion if a little tissue is removed from the insertion on the first cut. Thus it is rather easy to obtain up to 28 bleedings from the same animal within a short time. The blood is allowed to run from the nail to the slide concavities. Blood can be collected with a capillary pipette from the slide or directly from the cut. Bleeding is stopped by cauterizing with the small metal spatula or similar device.

The Free Energy of Hydrolysis of *p*-Nitrophenyl Phosphate[1]

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"Phosphotransferase" activity, which is found associated with certain phosphatase preparations, involves the transfer of phosphate from certain donor phosphates to suitable acceptors. We have previously reported that with some acid phosphatases of plant and animal origin, *p*-nitrophenyl phosphate can serve as a donor (*1*). Meyerhof and Green (*2, 3*) have more recently disclosed that the alkaline phosphatase prepared from intestinal mucosa also possesses phosphotransferase activity and can utilize as donors phosphocreatine, glucose-1-phosphate, and phosphoenolpyruvate as well as *p*-nitrophenyl phosphate. We have not been able to demonstrate any evidence of transfer with these first three substrates when employing citrus or alfalfa phosphotransferase. Of these substrates, only phosphoenolpyruvate was significantly hydrolyzed, and that but slightly.

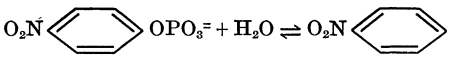
Meyerhof and Green suggest that a correlation exists between the effectiveness of a donor with the $-\Delta F^\circ$ of its phosphate bond, in the case of the three nonaryl esters. They found nitrophenyl phosphate to be an excellent donor, and if their suggestion is correct this compound should have a high free energy of hydrolysis. It is the object of this paper to report the determination of the $-\Delta F^\circ$ of hydrolysis of nitrophenyl phosphate.

The determination was made by measuring the equilibrium constant of the hydrolysis of nitrophenyl phosphate. With even a moderately low value of $-\Delta F^\circ$, the equilibrium concentration of nitrophenyl phosphate would be so low as to evade measurement by ordinary phosphate determinations. However, with P^{32}-labeled phosphate it becomes relatively easy to measure the nitrophenyl phosphate formed, after isolating it by carrier nitrophenyl phosphate.

Reaction mixture A: This consisted of 65 ml of an aqueous solution, 0.332 *M* with respect to *p*-nitrophenol, 0.123 *M* with respect to P^{32}-labeled K_2HPO_4, and containing 40 mg of a commercial alkaline phosphatase prepared by the method of Schmidt and Thannhauser (*4*). The pH was 8.95. *Reaction mixture B:* This was the same as A, except that the enzyme was omitted. Reaction time, 72 hr; temperature, 38° C.

The synthesized ester was isolated along with added carrier (0.500 g disodium *p*-nitrophenyl phosphate · 2 H_2O) after first removing inorganic phosphate as $Ba_3(PO_4)_2$. The ester was obtained by barium precipitation with 5 volumes of ethanol. Three crystallizations were sufficient to give constant activity.

The equilibrium constant was calculated for the hydrolysis written in the following way:



and it is expressed in terms of total forms of each substance (without regard to ionic form) and its dissociation constant.

$$K = \frac{(P_t)\ (A_t)}{(E_t)\ (H_2O)} \times \frac{(H^+)}{(H^+) + K_A} \frac{\frac{(H^+)^2}{k'_B\, k''_E} + \frac{(H^+)}{k''_E} + 1}{\frac{(H^+)^2}{k'_P\, k''_P} + \frac{(H^+)}{k''_P} + 1},$$

where P_t refers to the total concentration of phosphate, and k'_P and k''_P to the first and second ioniza-

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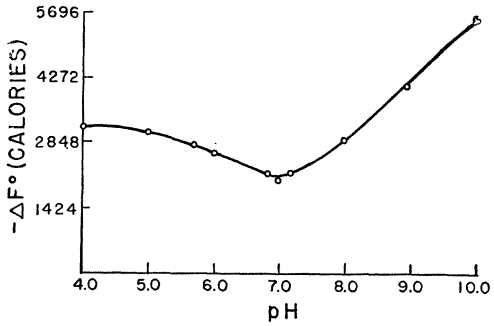

FIG. 1. Apparent "phosphate bond energy" of *p*-nitrophenyl phosphate.

tion constants of phosphoric acid, respectively. Similarly, E_t, k'_E and k''_E have corresponding meanings with respect to nitrophenyl phosphate and A_t and k_A with respect to nitrophenol.

As an approximation, concentrations are employed instead of activities. The concentration of water is assumed to be that of pure water. Following the convention of Lewis and Randall (*5*), the standard state of water is taken as pure water. pK values are not corrected to reaction temperature from their values as given in the literature. The following values were employed:

$$pK'_P = 1.97 \ (6), \ pK''_P = 6.82 \ (6), \ pK'_E = 2^3, \quad pK''_E = 5.7 \ (7), \ pK'_A = 7.18 \ (8).$$

The standard free energy of the above reaction is given by

$$\text{I} \quad \Delta F^\circ = -RT\ln\frac{(P_t)\ (A_t)}{(E_t)} - RT\ln \left(\frac{(H^+)}{(H^+)+K_A}\right)\left(\frac{\frac{(H^+)^2}{k'_E\,k''_E}+\frac{(H^+)}{k''_E}+1}{\frac{(H^+)^2}{k'_P\,k''_P}+\frac{(H^+)}{k''_P}+1}\right)$$

It is not necessary to know the actual concentrations of P_t and E_t but only their ratio which, as a sufficiently good approximation, is equal to total radioactivity of the reaction mixture divided by the radioactivity found in the nitrophenyl phosphate. The value found in the reaction mixture was 2.135×10^6 cpm. In Expts A and B the nitrophenyl phosphate contained 732 and 1,064 cpm, respectively. The average ΔF° is $-1{,}615$ cals. This value refers to the reaction as written and is independent of pH.

It is common practice to evaluate the so-called free energy of hydrolysis of phosphate esters by the use of the "apparent equilibrium constant" (e.g., see Alberty *et al.* [*9*]), in which only the total concentrations of the reactants are considered. Such an expression corresponds to the first term in the right side of equation I. The "apparent ΔF°" is proportional to the r'P of Dixon (*10*). Fig. 1 shows the dependence of the "apparent ΔF°" on pH. The latter value is only moderately high at pH 4, namely, $-3{,}180$ cal; it drops slowly to a minimum of about $-2{,}200$ cal at pH 7.0, and then increases to higher values at the rate of $-1{,}424$ cal/pH unit.

The efficacy of a donor depends upon enzyme specificity with regard to the donor phosphate, the acceptor and its phosphate, as well as on energy considerations. Thus "bond" energies are only permissive in the matter of transferability. In the present case the "apparent ΔF°" values do happen to accord with our observation that efficacy of transfer in the acid region increased with decreasing pH (*1*).

[3] The primary dissociation constant of *p*-nitrophenyl phosphate is too low to permit its determination with a glass electrode. It is reasonable to assume that it is lower than k'_P, and by analogy with other phosphate esters the value cited above is probably of the right magnitude. However the difference in ΔF° occasioned by this uncertainty is inconsequential in the pH range for which calculations are shown.

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Reactions of Dibenzodixanthylenes

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Dixanthylene (I) is almost colorless at low temperatures, but acquires a bluish-green color on heating, and the hot solutions—for example, in anisole—are also bluish-green (*1*). The authors have studied the thermochromic behavior of the two dibenzodixanthylenes (II and III) and have found that (II) is pale-yellow at room temperature, green when molten, mp, 345° C, gives a bluish-green solution in warm anisole, and in general exhibits remarkable thermochromic behavior. (III), which forms yellowish-green crystals, melts at 295° C, forming an olive-green melt. Its solutions in anisole are more weakly thermochromic to the naked eye than solutions of (II). In both cases, (II) and (III), the reversibility of the thermochromic phenomenon is the same as in the case of dixanthylene (*2*).

(II) and (III) have been obtained by the action of copper bronze on 1: 2-benzoxanthone or 3: 4-benzo-