1,4-Bis-(isopropylamino)-anthraquinone.—A commercial sample was similarly purified to give material, m.p. 179–180°.

1,4-Bis-(β-hydroxyethylamino)-anthraquinone.—Leucoquinizarin in excess ethanolamine was heated at 100° for 3–4 hours. The product, which had separated out as fine needles, was washed with water, dissolved in pyridine and precipitated into 2% HCl, then recrystallized from 2-methoxyethanol to yield needles, m.p. 235–236°. A second crystallization gave material, m.p. 242.5–244.0°.[9]

1,4-Bis-(β-acetamidoethylamino)-anthraquinone.—A solution of 5.4 g. (0.052 mole) of β-acetamidoethylamine[10] and 6.25 g. (0.026 mole) of leucoquinizarin in 40 ml. of 1-butanol was refluxed under nitrogen for 5 hours. The red-brown solution was oxidized by pouring into a solution of 2 g. of sodium persulfate in 100 ml. of water and 25 ml. of ethanol and letting stand under a current of air overnight. The semisolid residue which remained after evaporation was washed with water and crystallized from methanol to give 1.9 g., m.p. 213–217°, and 2.9 g., m.p. 210–213°; total crude yield 4.8 g., 45%. Recrystallization from methanol raised the melting point to 218–219°, small dark blue needles.

1,4-Bis-(γ-aminopropylamino)-anthraquinone.—A solution of 2.4 g. of leucoquinizarin in 10 ml. of 1,3-diaminopropane was heated for 30 minutes at 100°, then air was bubbled through for another 10 minutes; 30 ml. of water was added and the mixture allowed to cool. The resulting crystalline mass was filtered, then washed thoroughly with water. Recrystallization from petroleum solvent (b.p. 90–120°) yielded the 1,4-bis-(γ-aminopropylamino)-anthraquinone, m.p. 135.5–137.0°, which was converted into the dihydrochloride, m.p. 251–253°, for analysis.

1,4-Bis-(β-aminoethylamino)-anthraquinone.—A solution of 9.6 g. (0.04 mole) of leucoquinizarin in 50 ml. of ethylenediamine was heated 1 hour at 100°, then air was bubbled through the hot solution for 3½ minutes and the mixture let stand overnight at room temperature. The crystalline product which had separated was filtered, washed with a small amount of ether and hot methanol to yield 3.6 g. of copper-colored crystals, m.p. 204–204.5°. Another 0.4 g., m.p. 199–201°, was isolated from the methanol wash solution to give a total of 4.0 g., 41%, crude product. Recrystallization from butanol gave 80% recovery of black crystals with a copper reflex, m.p. 207–208°.

(9) Y. Bansho, *J. Chem. Soc. Japan, Ind. Chem. Sect.*, **55**, 666 (1952); *Chem. Abstr.*, **48**, 6701d (1954), reports m.p. 239°.

(10) S. R. Aspinall, *J. Am. Chem. Soc.*, **63**, 853 (1941).

1,4-Bis-(β-N-ethylaminoethylamino)-anthraquinone.—A mixture of 2.4 g. (0.01 mole) of quinizarin, 0.5 g. of sodium carbonate 3.4 g. (0.036 mole) of phenol and 8 ml. of water was swept out with nitrogen, and 3.1 g. (0.035 mole) of β-(N-ethylamino)-ethylamine in 12 ml. of ethanol was added. The mixture was heated under nitrogen at 100° overnight, then precipitated into a saturated sodium chloride solution. The oily product was dissolved in acetone and reprecipitated into saturated sodium chloride solution. The product, still oily, was dissolved in a small amount of ethyl acetate, diluted with benzene, and chromatographed on alumina. Elution with ethyl acetate yielded 0.5 g. of dark needles, m.p. 115–120°. Crystallization from hexane raised the melting point to 120.5–122°.

1,4-Bis-(β-N-diethylaminoethylamino)-anthraquinone.—A mixture of 2.4 g. (0.01 mole) of quinizarin, 4.1 g. (0.035 mole) of β-N-diethylaminoethylamine, 0.5 g. of sodium carbonate, 3.4 g. (0.036 mole) of phenol, 12 ml. of ethanol and 8 ml. of water was refluxed in a nitrogen atmosphere for 6 hours. Spectra of the reaction solution were taken every hour, and the reaction was stopped when the characteristic spectrum remained constant for 2 hours.

The product was isolated by precipitation into saturated sodium chloride solution, reprecipitating from acetone solution into sodium chloride solution two times, from Methyl Cellosolve solution by addition of a small amount of water, and crystallization from hexane to yield 2.1 g., 48.0%, m.p. 109–110°, glistening purple platelets with a red reflex. Chromatography from hexane on alumina followed by elution with chloroform gave a 70% recovery of dark blue radiating needles with a violet reflex, m.p. 111.5–112°. Two more crystallizations from hexane gave dark needles with a purple reflex, m.p. 112–112.5°.

Acknowledgment.—We wish to thank Mr. J. F. Downey and Dr. A. D. Jarrett for the samples of 5,8-dihydroxy-1,4-bis-(β-aminoethylamino)-anthraquinone, Dr. S. M. Bloom for the samples of 1,5-bis-(β-aminoethylamino)-anthraquinone and 1,5-bis-(β-diethylaminoethylamino)-quinone, Mrs. R. W. Fajer for the sample of 1,5-(γ-aminopropylamino)-anthraquinone, and Dr. S. Dershowitz for the sample of 1,5-bis-(β-amino-α,β-dimethylethylamino)-anthraquinone. We also thank Dr. S. G. Cohen for informative discussions.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL SCIENCES, PURDUE UNIVERSITY, LAFAYETTE, IND., AND THE BIOLOGY DEPARTMENT, BROOKHAVEN NATIONAL LABORATORY, UPTON, N. Y.]

An All-or-None Assay for Assessing the Role of Amino Acid Residues in Enzyme Action—Application to Phosphoglucomutase[1,2]

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An "all-or-none" assay, which distinguishes fully active and partially active enzymes from inert enzymes in a mixture of all of these, is described. Unlike the usual "efficiency" assay, the "all-or-none" assay gives equal weight to active and partially active enzymes. The difference in the two assays is exploited in interpreting the effect on phosphoglucomutase of the methylene blue-catalyzed photooxidation reaction, and provides evidence for a formation of partially active enzyme intermediates, probably *via* oxidation of a single "surface" histidine residue. A correlation of the activity loss as determined by the "all-or-none" assay with previous data on amino acid modification during photooxidation indicates that production of an inert enzyme having an efficiency less than $1/_{200}$ of the native enzyme parallels the modification of a single surface methionine residue. The general applicability of such "all-or-none" assays to enzymes which form a measurable intermediate of reasonable stability is discussed.

Introduction

Methods utilizing chemical modification as a means of identifying amino acid residues involved in enzyme action have many pitfalls, not the least of which arises from the possibility of producing a partially active enzyme by virtue of the modification reaction under study. For example, if the observed activity of an enzyme has been reduced 10-fold by treatment with a given reagent, the remaining activity might be the result of (a) a small amount (10%) of fully active enzyme in the presence of inert enzyme (90%), (b) the reduced efficiency of a modified enzyme, 10% as active as the native enzyme and (c) complex mixtures of active, partially active and inert enzyme. To aid in the detection and analysis of such situations, an "all-or-none" assay has been devised which, in conjunction with the conventional "efficiency" assay, may also be used to determine whether modification of a given residue produces partially active or inert enzyme.

The conventional "efficiency" assay for assessing enzymic activity measures the amount of substrate converted, or product produced per unit time, under standard conditions. It therefore gives a weighted average of the catalytic efficiencies of the various enzymic species present in the assay. For example, an enzyme preparation which, by virtue of chemical modification, contained fully active enzyme (15%),

(1) A preliminary account of some of this work has been published.[2]

(2) W. J. Ray, Jr., J. J. Ruscica and D. E. Koshland, Jr., *J. Am. Chem. Soc.*, **82**, 4739 (1960).

modified enzyme with 10% of its native activity (30%) and inert enzyme (55%), would by such an "efficiency" assay be 18% as active as an equivalent amount of native enzyme.

On the other hand, an "all-or-none" assay, as herein defined, is designed to measure the *number* of enzymic sites possessing a pre-determined minimum efficiency rather than the *rate* of product formation. This is accomplished by quantitating some property, which is an essential feature of the over-all assay reaction, after a sufficient time interval has elapsed to ensure that the time dependence of this property has essentially become zero, even with respect to enzymic species that are able to function only at greatly reduced efficiency. For example, all the phosphoglucomutase in a complex mixture of active, partially active and inactive enzyme which retained its ability to transfer its phosphate to a substrate could be measured in such an assay by (a) using phosphoglucomutase previously labeled with P^{32}-phosphate, (b) treating the enzyme mixture to be assayed with a large excess of substrate (allowing sufficient time for all the enzyme capable of phosphate transfer to react), (c) determining the amount of phosphate remaining attached to the enzyme. Only inert enzyme would fail to lose its phosphate label in such an assay, and in the case cited above where 18% of the initial activity would be observed by the conventional "efficiency" assay, the "all-or-none" assay would indicate that 45% of the enzyme retained at least a small fraction of its original catalytic activity.

Thus, in such cases, the difference in enzyme activity remaining, as determined by these two assays, depends on the difference in the quantity measured. By exploiting this difference in conjunction with the measured modification rates of the various amino acid residues, the relative importance to the enzyme of individual amino acid residues may be assessed. In the present paper the application of this approach to the chemical modification produced by methylene blue-sensitized photoöxidation of phosphoglucomutase will be described.

Experimental

Reagents and Procedures.—The apparatus and general procedure for the photoöxidation reaction have been described previously.[3] Phosphoglucomutase was prepared from rabbit muscle by a modification of the method of Najjar.[4] The efficiency assay, measuring the rate of conversion[5] of G-1-P to G-6-P during a 10-minute time interval, utilizes conditions similar to those of Najjar[4] and has been described in a previous publication.[3]

P^{32}-Labeled glucose phosphates were prepared by adding trace amounts of sucrose phosphorylase and phosphoglucomutase to a solution, 10^{-3} *M* in sucrose and 10^{-4} *M* in $KH_2P^{32}O_4$, buffered at *p*H 6.0 with 0.1 *M* histidine. When the P_i had decreased to about 5% of its initial value, the reaction was terminated by passing the mixture through a small Dowex 50W-8% column in the acid form. The eluate, which contained G-1-P^{32} and G-6-P^{32} in an equilibrium ratio, was neutralized and subsequently used for labeling the enzyme.

P^{32}-Labeled phosphoglucomutase was prepared by treating a mixture of labeled glucose phosphates (above) with a 10-fold excess of enzyme at 0° for a few minutes, at *p*H's varying from 6.0 to 7.5. No added cofactors were required. Residual P^{32}-labeled substrate was removed either by chromatography,[3] or by a batch-wise procedure in which the enzyme in 10^{-3} *M* phosphate buffer, *p*H 6.0, was absorbed on a 2 × 2 cm. carboxymethylcellulose column, previously equilibrated against the same buffer. After washing the column with the dilute buffer until the radioactivity in the effluent had reached an insignificant value, the enzyme was eluted with a small volume of 0.05 *M* phosphate buffer, *p*H 7. Up to 90% of the substrate radioactivity could thus be incorporated into the enzyme.

All-or-None Assays.—All-or-none assays were carried out in three ways: (I) by measuring loss of P^{32}-phosphate from P^{32}-labeled enzyme on treatment with G-1-P, (II) by measuring loss of P^{32}-phosphate from P^{32}-labeled enzyme in treatment with G-6-P, (III) by measuring labeling of the enzyme on treating unlabeled enzyme with a mixture of G-1-P^{32} and G-6-P^{32}.

Assays I and II, which involved treatment of photoöxidized samples of P^{32}-labeled enzyme with 0.01 *M* G-1-P or G-6-P, were carried out at 30° for 5 minutes in 0.07 *M* potassium hydrogen phthalate buffer, *p*H 5.5, with an enzyme concentration of 0.1 mg./ml. After addition of 1 mg./ml. of carrier protein (bovine serum albumin) and trichloroacetic acid to a final concentration of 5%, the tubes were allowed to stand for 2 hours in ice. Aliquots of the supernatant liquid were then assayed, both for total P^{32} content and also for acid-labile phosphate, using the isobutyl alcohol extraction procedure for P_i. Other aliquots were subjected to paper electrophoresis (pyridine–acetic acid–water, 150:6:1350) for 2 hours at 900 v. As a control, phosphoglucomutase was used which had been photoöxidized until no detectable enzyme activity remained by the efficiency assay (40 minutes). When such material was used in all-or-none assays I and II, about 2% of the total radioactivity appeared in the trichloroacetic supernatant. This quantity was subtracted from the measured value of each assay in these series as a "constant" which was attributed to traces of P^{32}-glucose phosphates in the labeled enzyme preparation and to incomplete precipitation of the protein under the conditions employed.

Assay III, in which unlabeled enzyme, previously subjected to photoöxidation, was treated with radioactive substrate, was carried out in a solution 1.2×10^{-3} *M* in $MgSO_4$, 1.0×10^{-3} *M* in P^{32}-labeled glucose phosphates (an equilibrium mixture of G-1-P^{32} and G-6-P^{32}), 0.05 *M* in histidine, and 0.01 *M* in trishydroxymethylamine at *p*H 7.4, with sufficient enzyme to incorporate *ca.* 15% of the total radioactivity into the protein. (The amount of enzyme, *i.e.*, the dilution of photoöxidized enzyme required for this degree of incorporation, was determined by trial and error in pilot runs.) In each case, serum albumin was added to the assay mixture in sufficient amounts to maintain a protein concentration of 0.1 mg./ml. After 25 minutes at 30°, additional serum albumin and trichloroacetic acid were added as above and the mixture allowed to stand for 2 hours in ice. After centrifugation and washing (5% trichloroacetic acid) the protein was dissolved in 0.1 *N* NaOH and reprecipitated with trichloroacetic acid. The second precipitate was subsequently dissolved in 6 *N* ammonium hydroxide and plated for counting. As a control, phosphoglucomutase was used which had been photoöxidized 40 minutes. When such material was used in this assay, only about 0.3% of the total radioactivity was carried down by the protein. The quantity was subtracted from each assay tube as a constant which was attributed to non-specific adsorption by precipitated protein.

Results and Discussion

Comparison of Efficiency and All-or-None Assays.—The effect of dye-sensitized photoöxidation on phosphoglucomutase, as determined by the efficiency assay and all-or-none assay I, is shown in Fig. 1. At all points, the quantity registered, relative to the same quantity at $t = 0$, is substantially greater by the all-or-none assay than by the efficiency assay. This difference results from the fact that these assays were designed to take advantage of different aspects of phosphoglucomutase action[6] (eq. 1 and 2) and therefore measure different quantities.

$$\text{G-1-P} + \text{EP} \rightleftharpoons \text{G-1,6-P}_2 + \text{E} \qquad (1)$$

$$\text{G-1,6-P}_2 + \text{E} \rightleftharpoons \text{G-6-P} + \text{EP} \qquad (2)$$

The efficiency assay measures the rate of conversion of G-1-P to G-6-P in the presence of catalytic amounts of enzyme. A decrease in the efficiency of the enzyme will therefore be reflected in a decreased amount of G-1-P converted in the limited assay interval. On the other hand, all-or-none assay I (and also II, *vide infra*) measures the amount of enzyme which retains its capacity to participate in a phosphate transfer reaction with substrate even when the efficiency of this reaction is low. The labeled enzyme in the latter case is measured directly, rather than indirectly (*i.e.*, by assessing its catalytic capabilities), and its reaction

(3) W. J. Ray, Jr., and D. E. Koshland, Jr., *J. Biol. Chem.*, **237**, 2493 (1962).

(4) V. A. Najjar, in "Methods in Enzymology," ed. by S. P. Colowick and N. O. Kaplan, Vol. 1, Academic Press, Inc., New York, N. Y., 1955, p. 294.

(5) Abbreviations: G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; G-1,6-P_2, glucose-1,6-diphosphate; P_i, inorganic phosphate; P_{10}, labile phosphate, *i.e.*, phosphate produced on 10-min. hydrolysis in 1 *N* acid at 100°; EP, phospho-form of phosphoglucomutase; E, dephospho enzyme.

(6) V. A. Najjar and M. E. Pullman, *Science*, **119**, 631 (1954).

efficiency enters only in limiting cases. Thus if native enzyme were able to transfer completely its phosphate to G-1-P in one second, an assay time of 17 minutes (*i.e.*, 1000 seconds) would be sufficient for a modified enzyme operating at 1/1000th the efficiency of native enzyme to undergo the same reaction. By labeling the phospho-enzyme with P^{32}-phosphate the extent of phosphate transfer can be measured quantitatively, and by using a 17-minute assay interval, an enzyme whose efficiency was lowered by a factor of 1000 would thus register in the all-or-none assay to the same degree as native enzyme. Only inert enzyme would retain its radioactive label and fail to register.

The term "inert" is used here to refer to enzyme which does not lose its P^{32}-label under the designated conditions of the various all-or-none assays. Enzyme with zero activity would of course be inert, but enzyme with a very low efficiency might also fail to register in the assay. In the above example, enzyme with an activity reduced by 10^{-6}-fold relative to the native enzyme would thus fail to undergo phosphate transfer in this assay. Hence inert as used here means not detectable under the assay conditions. If these assay conditions are correctly designed the detectable activity limits can be extremely low, but inert or "zero activity" in an enzyme system must always tacitly assume the phrase, "within experimental error."

Since the measured turnover number (G-1-P to G-6-P conversion) under the conditions of all-or-none assay I was 100 min.$^{-1}$ per 77,000 grams with native enzyme and since a positive effect in this assay might result from only a part of one turnover, the conditions utilized for this assay would allow at least 90% of a sample of native enzyme to undergo at least one phosphate transfer in 1/200th of the total time. Thus, a partially active enzyme with a 200-fold reduction in efficiency (*i.e.*, a turnover number of 0.5 min.$^{-1}$) would have a 90% probability of participating in at least one phosphate transfer and therefore registering in the assay. Enzyme which is inactive by this assay therefore must retain less than 0.5% of its original catalytic efficiency and may indeed be less active by several orders of magnitude.

Evidence for a Partially Active Species.—That the quantity measured at the various reaction times, relative to the same quantity at $t = 0$, was invariably greater by all-or-none assay I than by the efficiency assay (Fig. 1) is to be expected if a partially active enzyme species was produced by virtue of the modifying reaction. Since inert enzyme registers as zero in both assay systems, contributions from such enzyme were the same in both cases. However, partially active enzyme retaining at least 1/200 of its original activity would register as essentially 1.0 in the all-or-none assay but as <1.0 in the efficiency assay. Hence in the presence of partially active enzyme, the percentage remaining by the all-or-none assay should at all points be greater than the percentage remaining by the efficiency assay.

An alternate explanation for the differences observed in Fig. 1 is that the all-or-none assay depended only on the reaction of eq. 1 whereas the efficiency assay depended on the reactions of both eq. 1 and 2. If the modification preferentially eliminated the capacity of the enzyme to participate in step 2 but did not alter its capacity to participate in step 1, the results of Fig. 1 might be explained without invoking a partially active, modified enzyme, since phosphoglucomutase which can participate in only one step of the over-all two-step reaction mechanism is for all intents and purposes inactive as a catalyst. To test this possibility, all-or-none assay II, using G-6-P instead of G-1-P,

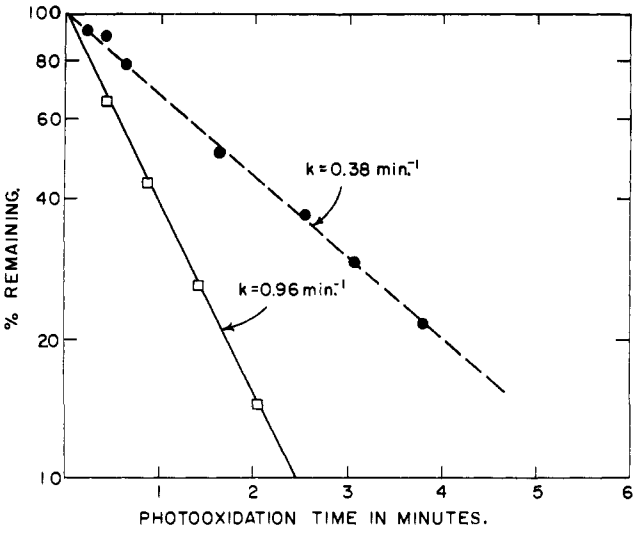


Fig. 1.—Effect of photoöxidation on the activity of phosphoglucomutase as measured by "efficiency" assay, □, and "all-or-none" assay (I), ●. The latter involves incubation of G-1-P with EP^{32}.

was run under the same conditions. In order for this test to be valid, however, it was necessary to make certain that a modified enzyme which had lost its capacity to transfer phosphate to G-6-P did not, when treated with G-6-P, transfer its phosphate to the small amount of G-1-P inevitably formed during the assay (assay II), and that a modified enzyme which had lost its capacity to transfer phosphate to G-1-P did not, when treated with G-1-P, also transfer its phosphate to the small amount of G-6-P formed in this assay (assay I). This would be impossible to demonstrate if the assay intervals employed were of sufficient duration so that equilibration of the P^{32}-label occurred between the 1- and 6-positions of glucose. A very short assay interval would have been required to preclude label shuffling with the relatively large amount of enzyme used in these assays under conditions of optimal enzymic activity, however. Assay conditions were therefore selected under which the intact enzyme was only about 1/100 as active as under optimal conditions (see Experimental), thus allowing the use of an assay interval of 5 min.

The following observations verify the essential absence of label equilibration in the glucose fraction under the conditions of these two assays: (a) Treatment of intact, P^{32}-phosphoglucomutase with a 3000-fold excess of either G-1-P or G-6-P (assays I and II) resulted in the transformation of at least 99% of the radioactivity to trichloroacetic acid soluble products. (b) Of the radioactivity in the trichloroacetic acid supernatant, 98% or more was found in the glucose phosphate region on paper electrophoresis, the remainder being found in the inorganic phosphate–glucose diphosphate region. (c) In assay I (treatment with G-1-P), 98% or more of total radioactivity in the trichloroacetic acid supernatant was acid stable, *i.e.*, was in the 6-position of glucose. (d) In assay II (treatment with G-6-P) 95% or more of the radioactivity was acid labile, *i.e.*, was in the 1-position of glucose. (e) Longer assay intervals invariably resulted in increased shuffling of label in the glucose phosphate fraction. (f) Points b, c, d and e applied equally well to samples of photoöxidized as well as intact phosphoglucomutase, the only difference being that successively smaller percentages of the total radioactivity appeared in the trichloroacetic acid supernatant with increasing photoöxidation time.

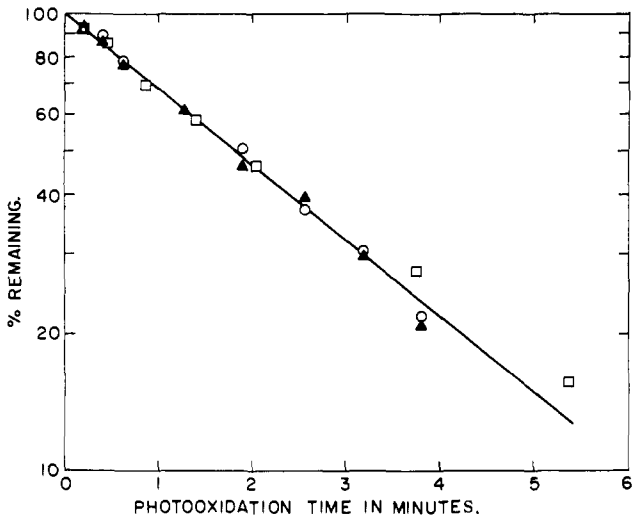


Fig. 2.—Comparison of "all-or-none" assays during photooxidation of phosphoglucomutase: assay I, EP^{32} and G-1-P, ○; assay II, EP^{32} + G-6-P, ▲; assay III, EP + E and G-1-P^{32} + G-6-P^{32} + G-1,6-P_2^{32}, □.

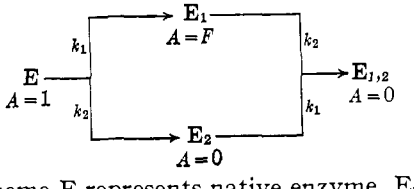
From these considerations it is evident that the position of the radioactive label in the trichloroacetic acid supernatant in both assays is the position in the glucose molecule to which it was first transferred. This is equivalent to saying that only a single phosphate transfer between substrate and enzyme is actually required to register in these assays.

The identity of quantities measured by assays I and II in Fig. 2 thus indicates that the photooxidation reaction affects at an identical rate the capacity of the enzyme to participate in both step 1 and step 2 of the over-all reaction mechanism. The difference in the fractional quantities measured by the efficiency and all-or-none assays must therefore be the result of a partially active enzyme produced by the modification reaction.

Evidence that the All-or-None Assay is Measuring an Enzymatic Process.—It might be argued that part of the phosphate transfer measured by all-or-none assays I and II was non-enzymatic, and was the result of the increased tendency, resulting from chemical modification, of the phosphate group in phospho-enzyme to undergo nucleophilic displacement. To test this point, all-or-none assay III was carried out using unlabeled enzyme which was treated with a mixture of G-1-P^{32} and G-6-P^{32} (containing trace amounts of G-1,6-P_2^{32} from the method of preparation). In such a system, dephospho-enzyme could be labeled in a one-step reaction with G-1,6-P_2^{32}, but there is no reason to believe that the enzyme used in these assays contained appreciable amounts of the dephospho-form, both from the method of preparation, and from Najjar's previous work.[6] Phospho-enzyme on the other hand must engage in two phosphate transfers in order to register, the first to remove the unlabeled phosphate group and the second to replace this phosphate by reaction with G-1,6-P_2^{32}. Time for completion of both processes was allowed in the assay. The fact that assay III, which requires at least two phosphate transfer steps, measures the same quantity (Fig. 2) as assays I and II, which require a minimum of only one transfer, indicates that an enzymatic process is indeed being studied in each of the all-or-none assays and that these assays are not measuring simply a non-enzymic capacity for release of phosphate by the enzyme.

Assay III is not only more complex, theoretically, than assays I and II, but it also requires more careful control of conditions. A large molar excess of radioactive substrate relative to enzyme in this assay would eliminate isotope dilution effects, but would make measurement of covalently bound enzymic phosphate very difficult, both because of increased soluble radioactivity (due to labeled substrate) and decreased amounts of phospho-enzyme (*i.e.*, increased amounts of dephospho-enzyme) owing to the equilibria in eq. 1 and 2. If a small excess of substrate is used, however, isotope dilution and equilibrium effects must both be considered. To minimize corrections, a variable dilution technique was employed which made it possible to maintain a reasonably constant ratio of active sites to substrate. Aliquots from the photooxidation reaction were thus diluted by trial and error in pilot runs until approximately 15% of the P^{32}-phosphate in a constant amount of substrate was incorporated by the enzyme in equal volumes of these dilutions. If ideally performed, *i.e.*, if the radioactivity incorporated by each time aliquot were exactly equal to that incorporated at $t = 0$, the relative numbers of catalytic sites undergoing phosphate exchange would then be proportional to the extent of the aliquot dilution required to achieve this situation. In practice, however, there was some deviation from this ideal situation and corrections both for equilibrium and isotope dilution must be applied. Fortunately these corrections tended to balance each other, as indicated in Table I and explained in the Appendix.

Correlation of Assays with Amino Acid Residues.—It now remains to correlate the results of Fig. 1 with the modification of individual amino acid residues by the photooxidation reaction. That the quantity measured by the all-or-none assays decreased during photooxidation less rapidly than the simultaneous rate of activity loss by the efficiency assay indicates, in the light of the foregoing section, that partially active species are indeed being produced by photooxidation. The fact that the quantity measured by the all-or-none assays actually decreases with time indicates that the partially active enzyme is itself being converted to an inert form of the enzyme by the photooxidation reaction. To identify the residues involved in these processes, *i.e.*, production of partially active and of inert enzyme, the method of kinetic analysis previously described[7] must be applied. General equations for inactivation *via* a partially active enzyme have been derived and are adequately represented by the scheme shown in eq. 3, which is consistent with the results shown in Fig. 1.



(3)

In this scheme E represents native enzyme, E_1, enzyme in which residue 1 has been modified, E_2, enzyme in which residue 2 has been modified, and $E_{1,2}$, enzyme in which both residues 1 and 2 have been modified, and constants k_1 and k_2 are the rate constants for modification of the amino acid residues, while A represents the specific activity of the various enzymic species and F is a fraction between 1.0 and 0. For completeness, modification of E_2 to yield $E_{1,2}$ is indicated in the above scheme, although such a conversion would have no influence on the activity assay. It would, however, be significant in the amino acid analyses.

(7) W. J. Ray, Jr., and D. E. Koshland, Jr., *J. Biol. Chem.*, **236**, 1973 (1961).

TABLE I

Photo-oxidation time, min.	Assay dilution	Enzyme[a] radioact., c./min.	Calcd.[b] [G-1,6-P_2]	Equil.[b] corrn. factor	Isotope[b] dil. corrn. factor	Over-all corrn. factor	$[EP]_i$	Act. sites remaining, %
0.00	10.0	6230	12,000	1.96	1.44	2.82	17,600	100.0
.44	8.2	6550	12,200	1.93	1.46	2.81	18,500	86.4
.87	6.0	7260	12,500	1.86	1.51	2.81	20,300	69.3
1.40	5.3	6970	12,400	1.88	1.48	2.78	19,500	58.5
2.04	4.0	7290	12,500	1.86	1.51	2.81	20,400	46.4
3.74	2.5	6830	12,300	1.90	1.48	2.81	19,200	27.4
5.38	1.3	7530	12,600	1.84	1.52	2.80	21,100	15.6

[a] Average of two duplicate aliquots; initial substrate radioactivity, 40,200 c./min. [b] See Appendix.

It follows from such a scheme that k_2 should be related to the slope of the all-or-none assay plot since this represents the rate at which the sum of the active and partially active enzyme ($E + E_1$) is converted to inert enzyme ($E_2 + E_{1,2}$). This rate constant is 0.38 in Fig. 1 which agrees very well with the figure 0.37 for the rate of oxidation of a single "surface" methionine residue determined from amino acid analyses under identical conditions.[3]

When partially active enzyme is produced by the chemical modification under study, the rate of activity loss (determined by the efficiency assay) will frequently be non-logarithmic (*i.e.*, non-linear on a semi-log plot, as in Fig. 3). However, the initial rate of activity loss in such cases will frequently be almost equal to the sum of the modification rates of those residues involved with the production of inert and partially active enzyme, respectively. The rate of decrease of the all-or-none assay, on the other hand, will be equal to the sum of the modification rates of only those residues producing inert enzyme. The difference between these rates should then approximate the modification constant of the residue producing partially active enzyme. The difference in rate constants in the present case (0.96–0.37, Fig. 1) is 0.6 $min.^{-1}$, which approximates the rate constant of a single surface histidine residue[3] and therefore constitutes *prima facia* evidence that the partially active enzyme is produced by modification of such a histidine residue of the enzyme.

It should be mentioned that the interpretation of the all-or-none assay is intimately related to the assumed mechanism of action of the enzyme. A change in the mechanism may or may not require a change in the conclusion derived from the all-or-none assay. For example, in the above analysis the Najjar mechanism has been assumed, but Cleland[7a] has suggested that certain kinetic observations of Bodansky[7b] are difficult to reconcile with this mechanism. It might well be asked what differences in interpretation of the all-or-none results would be required if the Najjar mechanism is modified. The answer would seem to be that the above interpretation is valid as long as the amount of phospho-enzyme produced is equal for all partially active species of enzyme. Thus, even such a drastic change in mechanism as one in which the phospho-enzyme appeared only as a side reaction and not an obligatory intermediate would require no reinterpretation of the all-or-none assay as long as this side reaction proceeded for all of the partially active species. If the phospho-enzyme is produced only as a side reaction, however, it is possible that modification of the enzyme might produce a species which could transfer phosphate only to G-1-P and not G-6-P. In this case the all-or-none assay could still provide valuable information in comparison to the efficiency assay, but the conclusions would be modified somewhat. The latter alternative does not seem likely for phosphoglucomutase, but the principle is important to bear in mind in the general application of the all-or-none assay (see below).

(7a) W. W. Cleland, *Biochim. Biophys. Acta*, **67**, 104 (1963).
(7b) O. Bodansky, *J. Biol. Chem.*, **236**, 328 (1961).

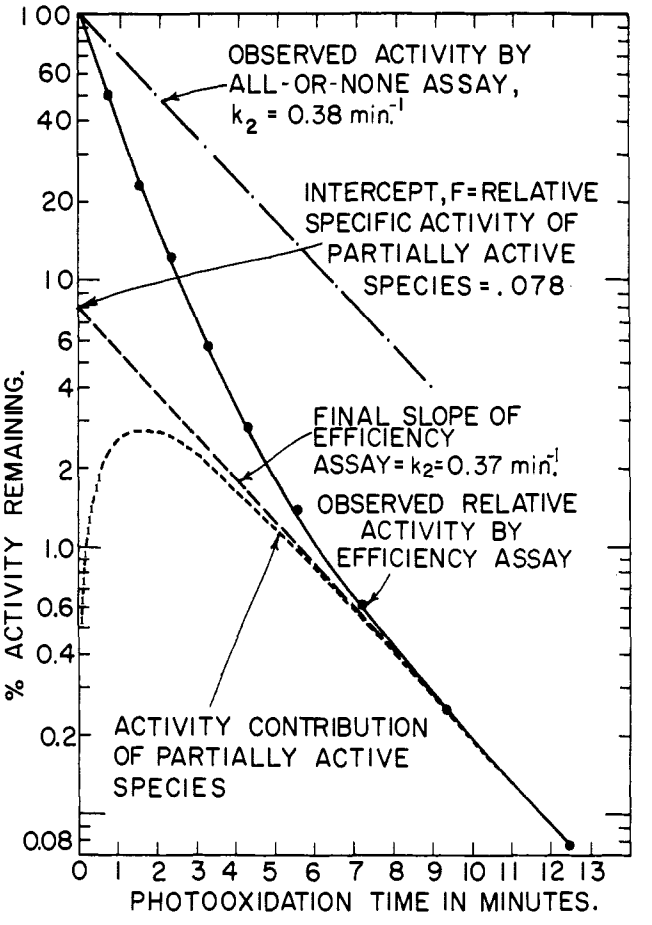


Fig. 3.—Comparison of final slope of "efficiency" assay curve with "all-or-none" assay. If the scheme of eq. 3 is valid, final slope represents rate of conversion of partially active enzyme to inert enzyme and, therefore, this slope equals $k_2/2.3$. "All-or-none" slope, shown by upper broken line, also represents k_2 as discussed in text. Lowest dashed curve represents calculated activity contribution of partially active enzyme during photooxidation assuming mechanism of eq. 3.

Comparison with Previous Results.—Figure 3 shows a plot of activity destruction during photooxidation extended to 99.9% loss of activity[3] followed by the efficiency assay as described previously. The decrease in rate of activity loss during the middle and latter phases of the reaction, *i.e.*, the decrease in slope of the semilogarithmic plot, has previously been attributed to the accumulation of a partially active enzyme according to the scheme shown in eq. 3. With the efficiency assay alone, however, the additional possibilities could not be eliminated that the non-linear activity loss in this plot was caused by (a) residual

intact enzyme resulting from a decreased photooxidation efficiency, (b) the presence in the enzyme sample of a structurally different mutase, or (c) a different type of enzyme which was positive in the efficiency assay[8] and oxidized more slowly than the phosphoglucomutase. These explanations may now be excluded, since (a) and (b) would result in a loss of catalytic efficiency (efficiency assay) equal to destruction of catalytic sites (all-or-none assay), while (c) would result in a loss of mutase sites at a rate exceeding the rate of destruction of catalytic activity. This leaves the activity loss scheme of eq. 3 as the remaining consistent explanation for the data represented by both Fig. 1 and 3.

The validity of this interpretation is further substantiated by the good agreement of the values of k_2 (eq. 3), determined by the all-or-none assays, with the previously obtained value for the production of inert enzyme on photooxidation, using an analysis of the data from the efficiency assay in Fig. 3. Since k_2 (0.37 min.$^{-1}$) by the latter method was obtained from the final slope of the activity destruction curve, *i.e.*, where the rate of formation of $E_{1,2}$ from E_1 was apparently being measured, whereas k_2 assessed by the all-or-none assays (0.36 to 0.38 min.$^{-1}$) was obtained by measuring the conversion of $E + E_1$ to $E_2 + E_{1,2}$, the concordance is strong support for the events depicted in eq. 3.

It is only fair to state that the identification of the individual residues of methionine and histidine with the respective steps in eq. 3 is somewhat more tenuous than the evidence for eq. 3 itself. The reason for this is that the constants for the amino acid residues were obtained by assuming certain groups of residues act as "surface" and others as "buried" residues. Until individual residue constants are obtained, these conclusions must be taken as working hypotheses. Recently, however, Schachter and Dixon[9] have measured the extent of reaction of individual residues in the photooxidation of a different enzyme (chymotrypsin) and their values coincided quite well with the values predicted from our previous kinetic analysis of the same system based on the concept of surface and buried residues.[10]

Finally it should be mentioned that the numerical value F (0.08) of the partially active species, which was obtained by analysis of the rate of decrease in activity with photooxidation time using the enzymic efficiency assay, is in agreement with the observations of the all-or-none assay. Thus, since an enzyme retaining only 0.5% of the efficiency of native enzyme would register as active in all-or-none assays I and II, it is obvious that a species which had retained 8% of its activity would register likewise, as observed.

General Applicability of the All-or-None Assay.—Although the type of all-or-none assays applied here is feasible only with the enzymes which form enzyme–substrate intermediates, the list of known enzymes which fall into this category is rapidly increasing. At present aldolase,[11] chymotrypsin,[12] trypsin,[12] phosphatase,[13] phosphoglyceric acid mutase,[14] transaldolase,[15] triosephosphate dehydrogenase,[16] thrombin,[17] elastase,[18] subtilisin,[19] acetoacetate decarboxylase,[20] deoxyribose phosphate aldolase[21] and 2-keto-3-deoxy-6-phosphogluconate aldolase[21] in addition to phosphoglucomutase have been shown to carry out their respective catalyses *via* such intermediates. Evaluation of the chemical modification of these enzymes using the present approach might well be feasible. This possibility has been recently substantiated by a preliminary account of the effect of dye-sensitized photooxidation of chymotrypsin.[10] A more detailed account of this work will be the subject of a forthcoming communication.

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Appendix

The total radioactivity incorporated into phosphoglucomutase under the conditions of all-or-none assay III is linearly related to the amount of enzyme active in the assay only over very narrow ranges of active enzyme/added substrate ratios. Corrections must therefore be made to relate the measured radioactivity incorporated to the amount of enzyme that reacts during the assay if this ratio changes appreciably. In order to minimize these corrections, the amount of enzyme used in the assay was varied by trial and error until almost the same ratio of active enzyme to added substrate was achieved as evidenced by incorporation of similar fractions of the total radioactivity of the added substrate by the enzyme, *i.e.*, about 15%. The required correction (to compensate for both equilibrium and isotope dilution effects) varied only slightly from sample to sample as indicated below.

Equilibrium Correction Factor.—Considering *only* the enzyme that was sufficiently active to exchange its label in the assay (*i.e.*, disregarding inert enzyme), $(EP)_i/(EP)_e$, the ratio of the phospho-enzyme initially present, to the phospho-enzyme remaining at equilibrium, may be calculated using Najjar's value of 3.8 for the equilibrium constant of eq. 2.[6] In these calculations, parentheses denote concentrations, brackets denote the total radioactivity of the enclosed quantity, and the subscripts i and e denote initial and equilibrium values of the indicated quantities. The assumption is also made that equivalent amounts of dephospho-enzyme and G-1,6-P_2 are always present, (*i.e.*, that $(E) = (G\text{-}1,6\text{-}P_2)$ and that $(EP)_i = (EP)_e + (G\text{-}1,6\text{-}P_2)_e$, since the enzyme is initially quite probably entirely phospho-enzyme as evidenced both from the method of preparation and from Najjar's work.[6]

In addition, the quantity of G-1-P present is neglected in all calculations. The following equations may then be written where [T] is the total added radioactivity, noting that the specific activity of the diphosphate is twice that of both EP and G-6-P.

$$[EP]_e\,[G\text{-}6\text{-}P]_e/[G\text{-}1,6\text{-}P_2]^2_e = 3.8/4 \qquad (4)$$

$$[EP]_e + [G\text{-}6\text{-}P]_e + [G\text{-}1,6\text{-}P_2]_e = [T] \qquad (5)$$

(8) Any enzyme which transformed G-1-P into an acid-stable phosphate would be positive.

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From eq. 4 and 5, the radioactivity of the G-1,6-P_2 at equilibrium, *i.e.*, $[G\text{-}1,6\text{-}P_2]_e$, may be calculated from the measured radioactivity of the added substrate and of the phospho-enzyme at equilibrium, and $(EP)_e/(EP)_i$ may then be represented as

$$(EP)_e/(EP)_i = [EP]_e/\{[EP]_e + 0.5[G\text{-}1,6\text{-}P_2]_e\} = 1/F_1 \quad (6)$$

Since $(EP)_e = [EP]_e/k_e$, the concentration of active phospho-enzyme initially present is given by eq. 7, where k_e is the specific activity of the exchangable phosphate at equilibrium and will be evaluated below.

$$(EP)_i = F_1[EP]_e/k_e \quad (7)$$

Values of F_1 calculated from eq. 6 are given in Table I as the equilibrium correction factor.

Isotope Dilution Correction Factor.—If the initial specific activity of added substrate is represented by k_i, the following equation may be written

$$k_e = k_i(G\text{-}6\text{-}P)_i/\{(EP)_i + (G\text{-}6\text{-}P)_i\} \quad (8)$$

After the appropriate manipulations this becomes

$$k_e = k_i\{[T] - [EP]_e - 0.5[G\text{-}1,6\text{-}P_2]\}/[T] = k_i/F_2 \quad (9)$$

Values of F_2 from measured values of [T] and $[EP]_e$ and calculated values of $[G\text{-}1,6\text{-}P_2]$ are given in Table I as the isotope dilution correction factor.

Over-all Correction.—From eq. 7 and 9, $(EP)_i = F_1F_2[EP]_e/k_i$. From Table I it may be seen that the product F_1F_2 is reasonably constant, in spite of variations in both F_1 and F_2. Thus to a good approximation $(EP)_i = k[EP]_e$. It might also be observed that the F_1F_2 product will be reasonably constant no matter what value is chosen for the equilibrium of eq. 2, and hence the validity of the present procedure is dependent only on the direct measurement of $[EP]_e$, since the ratio of $(EP)_i$ at any photoöxidation time to $(EP)_i$ at a reaction time of zero is the quantity actually under consideration. Thus if $X = \{[EP]_e + {}^1/_2[G\text{-}1,6\text{-}P_2]\}$, $F_1F_2 = X[T]/\{[T] - X\}$, which means that F_1F_2 is essentially constant over narrow ranges of X.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH OF THE UNIVERSITY OF WISCONSIN, MADISON, WIS.]

Studies on Polynucleotides. XVIII.[1] Experiments on the Polymerization of Mononucleotides. The Synthesis and Characterization of Deoxyguanosine Oligonucleotides[2]

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RECEIVED DECEMBER 11, 1962

In attempts to prepare deoxyguanosine oligonucleotides by polymerization of suitably protected deoxyguanosine-5′ phosphate, the following derivatives of the nucleotide were prepared: N,O$^{3'}$-diacetyl, N-acetyl, N-benzoyl, N-naphthoyl, N-di-*p*-methoxytrityl, O$^{3'}$-di-*p*-methoxytrityl and N,O$^{3'}$-bis-di-*p*-methoxytrityl. Only the di-*p*-methoxytrityl derivatives proved soluble in anhydrous pyridine, the medium used in all the previous work, but the polymerization was unsatisfactory. In search for alternative solvent systems, thymidine-5′ phosphate could be polymerized well in a mixture of pyridine and dimethylformamide in the presence of pyridinium Dowex-50 ion exchange resin. Using this procedure, N-acetyldeoxyguanosine-5 phosphate gave the expected homologous oligonucleotides which were separated on a DEAE–cellulose column and characterized before the removal of the N-acetyl protecting group from the guanine ring. Deoxyguanosine oligonucleotides obtained after removal of the N-acetyl-protecting group formed very large molecular weight aggregates having highly ordered secondary structure. This property of aggregation complicated the problem of characterization of the oligonucleotides by the standard chemical and enzymic methods.

In several previous papers from this Laboratory the polymerization of deoxyribomononucleotides to form homologous series of polynucleotides has been reported.[3–5] The method of polymerization was first developed for thymidine-5′ phosphate.[4] Because of the insolubility of other deoxyribonucleotides in dry pyridine, the only medium found satisfactory so far, and, of the reactivity of the amino groups, methods were developed for the selective protection of the latter groups in deoxycytidine aud deoxyadenosine nucleotides.[5] The resulting protected nucleotides served as suitable starting materials for the polymerization procedure and the protecting groups were removed under mild conditions before separation and characterization of the polymeric products.[5] The extension of this work to the polymerization of a protected deoxyguanosine-5′ phosphate did not prove straightforward and a modification of the polymerization procedure has now been developed. Furthermore, the characterization of the synthetic oligonucleotides by the chemical and enzymic methods used previously proved difficult until the discovery was made that the guanosine oligonucleotides were capable of forming large molecular weight aggregates with highly ordered secondary structure.[6] The present paper contains a detailed report of our experiments on the synthesis of these oligonucleotides, and, in particular, their isolation and characterization as the N-acetyldeoxyguanosine derivatives which are devoid of the property of aggregation and, therefore, are amenable to analysis by the methods developed previously.[4,5]

Acetylation of pyridinium deoxyguanosine-5 phosphate with a mixture of acetic anhydride and pyridine gave quantitatively N,O$^{3'}$-diacetyldeoxyguanosine-5′ phosphate[7] (I). It is interesting to note that the

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(2) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, the National Science Foundation, Washington, D. C.; and the Life Insurance Medical Research Fund, New York, N. Y.

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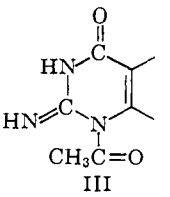
(7) The acetyl group in the ring is tentatively placed on the N-2-amino group. It is possible that it is on the 3-position (partial structure of the nucleotide III). It is improbable that the acetyl group is on O^6 or N-1-position, because spectrophotometrically an ionization around *p*H 10–11 can be demonstrated and this probably is due to the loss of a proton from N-1 position. The presence of an acetyl group at this portion of the molecule would preclude this ionization. The alternative that the acetyl group is on N-1 and that the ionization is due to the loss of a proton from the guanidine system involving the 2- and 3-position of the pyrimidine ring is considered unlikely.



III