paste tests. In the leaf dip and total spray tests, however, marked inhibition of growth resulted.

When a single leaf of young tomato seedlings was dipped in a 1% aqueous suspension of α-cyano-β-(2,4-dichlorophenyl) acrylic acid, the treated leaf died, but no other immediate effects on the rest of the plant were noted. Ten days later, the treated plants were only one third the height of the control plants. At the end of a month, the height of the treated plants was one half that of the control.

Death of the plants resulted when a 1% suspension of the compound was sprayed on young tomatoes. However, when a 0.1% suspension was used, growth inhibition occurred without visible tissue damage. In the latter experiment 2-in. tomato plants, growing one to a pot, were sprayed with 10 ml of a 0.1% aqueous suspension of α-cyano-β-(2,4-dichlorophenyl) acrylic acid, using 0.1% Tween 20 as the wetting agent. At the end of one week, the treated plants were noticeably smaller than the controls. Average measurements of height taken at the end of three weeks were: treated plants, 5.5 in.; control plants, 14 in. Although there was no tissue damage, and the color was normal, the treated plants exhibited formative effects, being unusually bushy with numerous axillary branches. The growth-inhibiting effect of α-cyano-β-(2,4-dichlorophenyl) acrylic acid, when applied to tomato plants at four different concentrations, is illustrated in Fig. 1.

Preliminary studies have indicated that, on a mole basis, the diethanolamine salt of α-cyano-β-(2,4-dichlorophenyl) acrylic acid is more effective than the free acid in inhibiting growth of tomato. This increased activity is probably due to the greater water-solubility of the salt.

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Crystalline and Amorphous[1] Insulin-Zinc Compounds with Prolonged Action

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The presence of zinc in pancreas and in crystalline insulin has given rise to a series of investigations on the interaction between insulin and zinc. As a result, protamine-zinc-insulin has become extensively used. The clinical results have been rather disappointing, for the weak initial action of this preparation has been very troublesome, especially in cases of severe diabetes.

In an effort to develop more suitable insulin preparations for single injection, we have carried out some combined chemical, biological, and clinical experiments, designed to elucidate the interaction between insulin and zinc.

It was first discovered that phosphate ions, which are used in the protamine-zinc-insulin preparation, are able to influence the physical-chemical relation between insulin and zinc.

In Fig. 1, *A* illustrates the solubility of insulin as a function of pH in phosphate buffer (an acid solution of insulin—40 u/ml—is adjusted to different pH values, and the dissolved insulin is determined spectrophotometrically). It can be seen that all the insulin is dissolved at the pH of blood, 7.3, which presumably explains why isoelectrically precipitated insulin does not possess a sustained effect. *B* shows the solubility of insulin in the same buffer, to which has been added the same amount of zinc as is found in protamine-zinc-insulin—2 mg/1000 u. The conditions for solubility

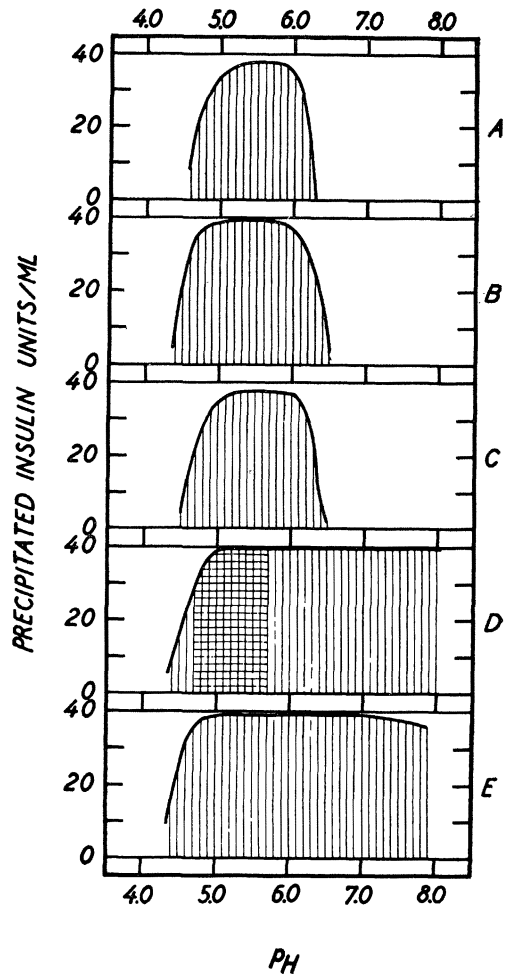

FIG. 1. Precipitation zone of insulin (40 u/ml) in: *A*, 0.01 mol sodium phosphate; *B*, 0.01 mol sodium phosphate with 2 mg zinc (as chloride)/1000 u; *C*, 0.01 mol sodium acetate; *D*, 0.01 mol sodium acetate with 2 mg zinc (as chloride) /1000 u; *E*, 0.01 mol sodium phosphate with 2 mg zinc (as chloride)/1000 u and 8.5 mg protamine/1000 u.

[1] Amorphous insulin, in this paper, refers only to the physical state of the insulin and not to the purity. The amorphous insulin is thus prepared by precipitation of dissolved crystalline insulin.

are altered only to a slight extent. *C* describes the solubility of insulin in acetate buffer. Only a slight deviation from the conditions in the phosphate buffer is seen, but on the addition of 2 mg zinc/1000 u there is a marked extension of the precipitation zone of the insulin, as shown in *D*. This and other experiments have established the fact that zinc has a great influence on the solubility of insulin as a function of pH in pure water, in acetate buffer, and in some other buffer solutions. The insulin is insoluble to the same degree as a zinc-protamine-insulin combination, as illustrated in *E*. Protamine (or other similar basic substances) is thus not a necessary factor for obtaining insolubility at the pH of blood. By using a small amount of zinc, as in protamine-zinc-insulin, insulin attains just as high or a higher degree of insolubility as in combination with protamine. The undissolved insulin contains chemically combined zinc. Phosphate buffer should not be used if this zinc effect is to be

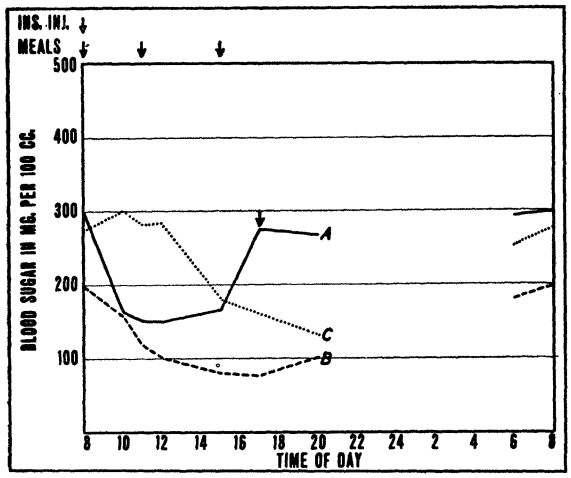

FIG. 2. Average blood-sugar curves (3–5 days) obtained with 3 depancreatized dogs. Average dose: 24 u. *A*, insulin-zinc (pH 2.7, 2 mg zinc/1000 u, solution. (Supplementary dose given at 5:00 P.M. when necessary.) *B*, amorphous insulin-zinc suspension (pH 7.2, 2 mg zinc/1000 u). *C*, protamine-zinc-insulin (NOVO) (pH 7.2, 2 mg zinc/1000 u).

obtained. The same applies to citrate buffer. Presumably the affinity of zinc for these substances is greater than for insulin.

Insulin preparations with the pH of blood, containing all the insulin in undissolved form, can thus be prepared by avoiding the presence of substances such as phosphate, citrate, ammonia, etc., which have a particular affinity for zinc. Our biological experiments, carried out at the same time on depancreatized dogs, clearly demonstrated that neither an acid solution of zinc and insulin (pH 3, 2 mg zinc/1000 u) nor an acid solution of protamine-zinc-insulin (protamine-zinc-insulin also contains 2 mg zinc/1000 u) possessed a sustained effect. This induced us to investigate whether the precipitated insulin-zinc had a prolonged action.

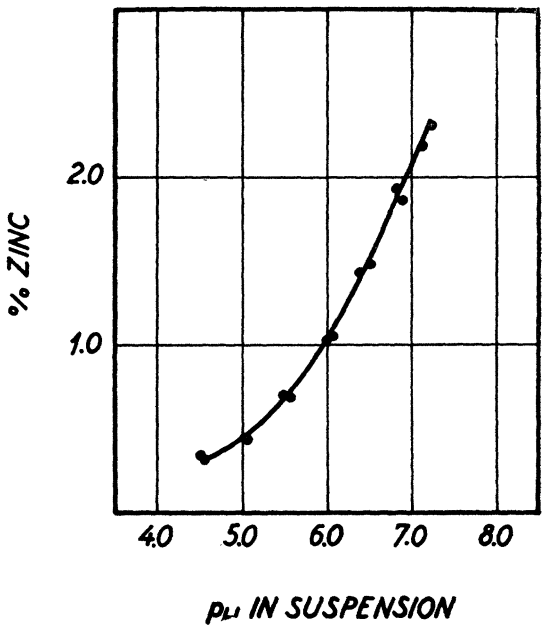

FIG. 3. Zinc content of insulin crystals (40 u/ml) suspended in 0.01 mol sodium-acetate with 2 mg zinc (as chloride)/1000 u as a function of pH.

The results clearly reveal a distinct prolonged effect of the undissolved insulin-zinc compound, as illustrated in Fig. 2, which shows a comparison between dissolved insulin and zinc (pH 3, 2 mg zinc/1000 u) and protamine-zinc-insulin (NOVO) (pH 7, 2 mg zinc/1000 u). The action of dissolved insulin and zinc ceases as early as 7–8 hr after injection, at which time both insulin-zinc and protamine-zinc-insulin exercise a pronounced effect. Fig. 1 *D* shows a squared area, which is the precipitation zone within which the amorphous insulin precipitate is completely or partly converted into a crystalline modification. Outside this zone the precipitate is stable and amorphous. The crystallization zone will vary somewhat with, for example, the amount of zinc, temperature, and time. Insulin crystals suspended in a solution of a zinc salt are preserved intact and completely undissolved in the pH range from 5 to 8, provided that substances which may interact with the zinc are not present. Other experiments have shown that the insolubility of amorphous and crystalline insulin caused by the presence of zinc demands an amount of zinc not less than approximately 0.5 mg zinc/1000 u.

Amorphous insulin and insulin crystals suspended in such zinc-containing media show, on analysis, a zinc content which is dependent both on the concentration of insulin and zinc used and on the pH value of the suspension. The relation between pH of suspension and the zinc content in the suspended insulin crystals is shown by the curve in Fig. 3. Insulin crystals, 40 u/ml, were suspended in solutions of various pH's, containing 2 mg zinc/1000 u and 0.01 mol sodium acetate. The increased zinc content of the crystals is not due to a simple precipitation of zinc hy-

droxide, as it also appears in a pH zone where the zinc hydroxide is not precipitated, just as a pronounced increase in the zinc content of the medium, within this pH range, does not give a corresponding increase in the zinc content of the crystals.

As described by Eisenbrand and Wegel (*1*), it has not been possible up to the present to prepare crystalline insulin with more than 0.8% zinc. However, from Fig. 3 it can be seen that insulin crystals under suitable conditions may contain more than 2% zinc.

Apparently the structure of the insulin crystals permits substances to enter into the crystals by diffusion and react chemically with the insulin within the crystal lattice. The insulin crystals with an increased zinc content are, contrary to the ordinary crystals, insoluble in water at the neutral point and retain their excessive zinc content, but if they are suspended in a neutral phosphate buffer they dissolve as the zinc is liberated and precipitate as zinc phosphate.

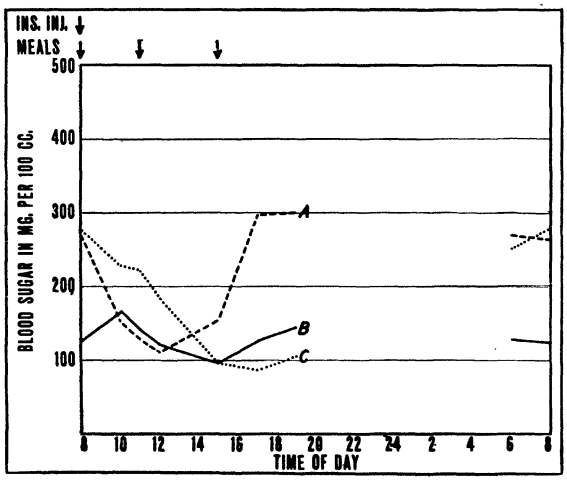

FIG. 4. Average blood-sugar curves (3–5 days) obtained with 5 depancreatized dogs. Average dose: 28 u. *A*, crystalline insulin NOVO, pH 2.7, solution; *B*, crystalline insulin-zinc suspension (pH 7.2, 1 mg zinc/1000 u); *C*, protamine-zinc-insulin NOVO (pH 7.2, 2 mg zinc/1000 u).

By increasing the zinc content of the crystals, not only the chemical but also the biological properties are altered. The action of ordinary insulin crystals suspended in water is not significantly different from that of ordinary dissolved insulin, as shown by Scott and Fisher, but in dog experiments with crystals of increased zinc content we found a striking prolongation of the insulin action.

Fig. 4 shows a comparison, on 5 depancreatized dogs, between (*A*) ordinary insulin (pH 3, 0.5 mg zinc/1000 u); (*B*) a suspension of insulin crystals with increased zinc content (pH 7, 1 mg zinc/1000 · u) —crystal size about 0.03 mm; and (*C*) protamine-zinc-insulin (pH 7, 2 mg zinc/1000 u). It appears that a suspension of these crystals possesses a markedly prolonged action, ensuring a low fasting blood sugar.

Up to the present, the action of the preparations containing amorphous or crystalline insulin-zinc and combinations of such preparations have been investi-

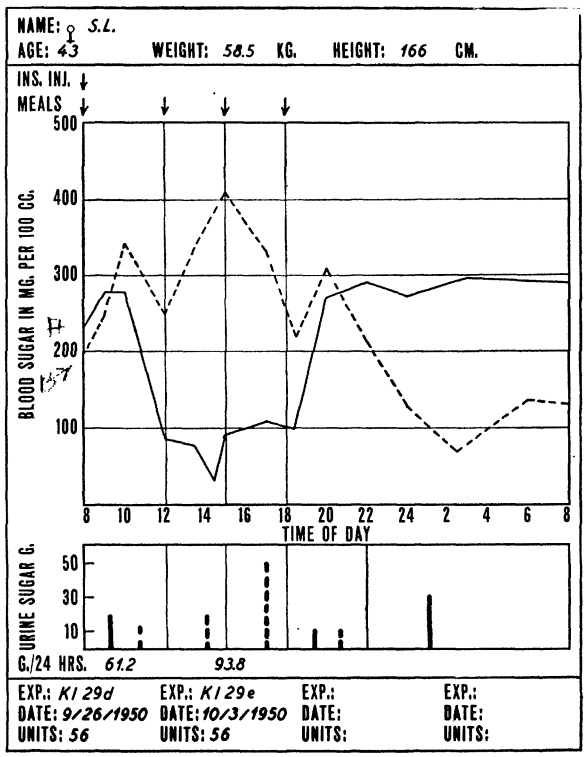

FIG. 5. Blood-sugar curves obtained with a diabetic, B. S. experimental method (*2*): *A*, crystalline insulin suspended in 0.9% sodium chloride solution, pH 6.0; *B*, crystalline insulin suspended in 0.9 sodium chloride solution, pH 6.0, 2 mg zinc/1000 u.

gated on a large number of diabetics.[2] The animal experiments were fully confirmed, and the chemical conditions for obtaining prolonged action were determined. With no exception a prolonged effect has been found in any preparation which contains, or will contain by adjusting the pH, all the undissolved insulin bound to zinc at the neutral point.

Fig. 5 shows, using the "B. S. experimental method,"[3] a comparison between two insulin preparations of the following composition: (*A*) insulin crystals suspended in physiological saline, 40 u/ml, pH 6.0, and (*B*) insulin crystals suspended in physiological saline containing 2 mg zinc/1000 u, 40 units/ml, pH 6.0. The insulin crystals suspended in water do not show any perceptible prolongation of action, in accordance with Scott and Fisher's results. On the other hand, if the suspension contains a small amount of zinc, 2 mg/1000 u (the same amount as in protamine-zinc-insulin), a pronounced retardation of the effect is obtained. The retarding effect of the

[2] Clinical investigations were performed at the Hvidøre Hospital, under the supervision of the chief physician, M. Jersild, whom we thank for permission to present illustrative graphs.

[3] The experimental technique described by Hallas-Møller (*2*) as the B. S. experimental method is, in brief: Under standard conditions (bed, a standard diet, regular-insulin-"preday") and at suitable intervals, 24-hr tests. In these 24-hr tests, one uses successively the insulin preparations it is desired to compare, so that they are characterized by a 24-hr blood-sugar curve as well as by excreted quantities of sugar.

zinc is not changed by decreasing the pH from 7 to 6, in spite of the parallel decrease in the zinc content of the crystals (Fig. 3). This and other experiments show that it is not a necessary condition for obtaining a prolonged effect that the crystals should possess an increased zinc content before the injection.

Fig. 6 shows (B. S. method) a comparison between

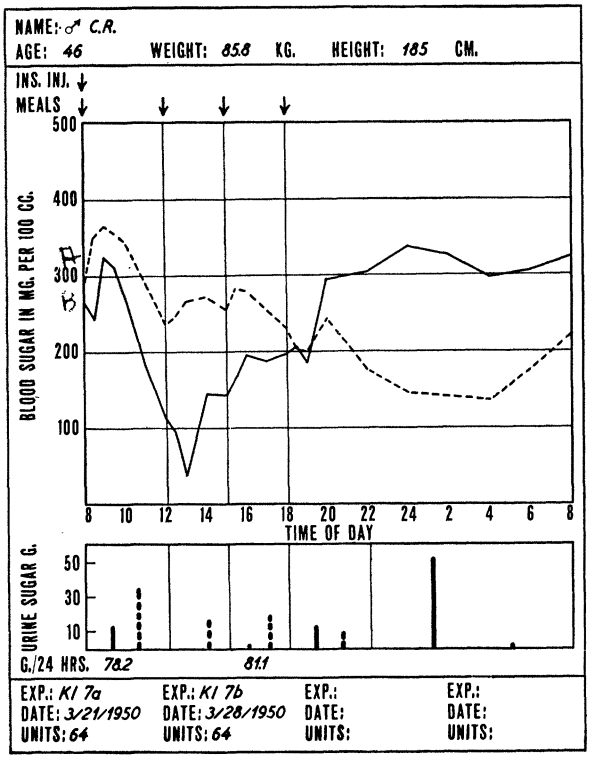

FIG. 6. Blood-sugar curves obtained with a diabetic, B. S. experimental method (2): *A*, crystalline insulin suspended in 0.01 mol acetate, pH 6.0, 2 mg zinc/1000 u; *B*, crystalline insulin suspended in 0.01 mol phosphate, pH 6.0, 2 mg zinc/1000 u.

(*A*) insulin crystals suspended in 0.01 mol sodium acetate buffer containing 2 mg zinc/1000 u, 40 u/ml, pH 6.0, and (*B*) insulin crystals suspended in 0.01 mol sodium phosphate buffer containing 2 mg zinc/1000 u, 40 u/ml, pH 6.0. The pH is 6.0, as the insulin crystals in phosphate-containing medium are dissolved at the neutral point (Fig. 1 B).

It appears from these experiments that the addition of phosphate in contrast to the addition of acetate completely neutralizes the protracted effect of an insulin crystal suspension containing zinc.

Fig. 7 shows single curves from 9 diabetics after injection of an insulin crystal suspension containing 2 mg zinc/1000 u (pH 7) and with a crystal size of about 0.01 mm. These curves clearly demonstrate the large range of activity of such a preparation.

The size of the insulin crystals used in the experiments (Figs. 4 and 7) is approximately 0.030 and 0.010 mm, respectively. Biological experiments point to the fact that preparations from crystals that are appreciably greater (0.075–0.1 mm) have a somewhat

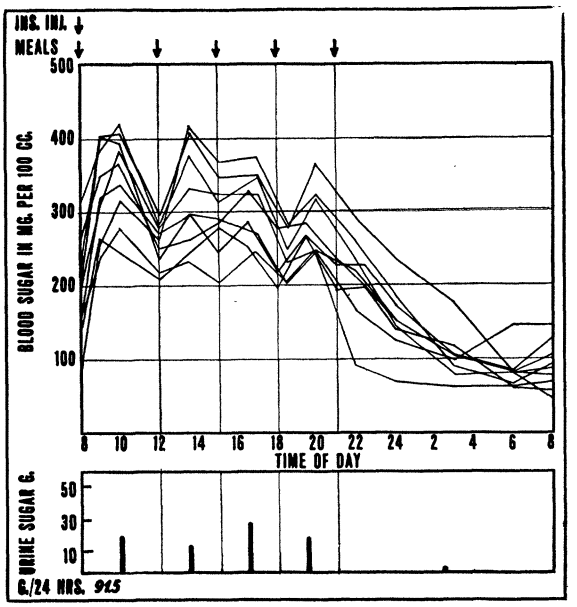

FIG. 7. Blood-sugar curves obtained from 9 diabetics; B. S. experimental method (2). Av dose: 55 u crystalline insulin-zinc suspension, pH 7.2, 2 mg zinc/1000 u.

more protracted action, just as, conversely, one obtains a somewhat faster action by grinding the crystals.

The following factors for the composition of the insulin-zinc preparations are essential for the biological effect: (1) The physical state of the insulin—amorphous, crystalline, large or small crystals; (2) the zinc concentration; and (3) the buffer. Owing to the fact that the insulin-zinc preparations are only based on the pure crystalline insulin and traces of zinc, they have so far never caused any allergic reactions.

The biological experiments here described are considered as preliminary steps in our efforts to develop insulin preparations with more suitable timing—i.e., a suitable adequacy between the initial and the retarded effect.

It is interesting to note that the retarding effect of zinc is exerted not only on insulin precipitated by protamine, globin, surfen, etc., but also on pure insulin alone which is made insoluble by combination with zinc. This is in agreement with, and supplements the findings of Scott and Fisher (*3*), who stated that the action of insulin precipitated by protamine or spermin was only significantly modified in the presence of small amounts of zinc. Other experiments, not reported here, have shown that other metals—e.g., the "crystallization metals," cadmium, cobalt, or nickel—can replace zinc. It is not yet possible to present the most suitable composition of the insulin-zinc preparations, as more clinical work is necessary.

In view of the fact that the organism, and especially the pancreas, contain zinc, the great influence of zinc on the insulin action may perhaps induce one to think of an *in vivo* relation between the two substances.

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The Reducing Potential of Illuminated Chloroplasts[1]

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The apparent limitation of the photolytic reaction to oxidants of standard potentials higher[2] than 0.0 v (*1, 2*), has prompted the publication of the following observations (*3*) on the reducing potentials that illuminated chloroplasts can attain. The redox potential that chloroplasts will finally establish with an oxidant in light is a reflection of the photoreducing intensity of the photolytic system. This potential was found to approach strongly reducing levels in washed chloroplasts freshly isolated, by the usual method (*4*), from leaves of Swiss chard. The isolated chloroplasts were suspended in distilled water and stored at 1° C.

In one type of experiment the final degree of reduction of methylene blue (MeBl) (at 10^{-5} *M*) by chloroplasts (at 3×10^{-6} *M* chlorophyll) was determined *in vacuo* photometrically, at pH 6.5, 15° C, and 1800 ft-c, a number of times over a period of several hours. The normal potentials (E_h) were then computed from the final percentages reduction of the dye, using the standard potential of 0.06 v for MeBl under the experimental conditions. The following positive, normal potentials were thus obtained from one series of determinations: at 1.5 hr after preparation of chloroplasts, 0.021 v; at 3.5 hr, 0.059 v; at 4 hr, 0.063 v; at 4.5 hr, 0.062 v; at 6.5 hr, 0.075 v; and at 9 hr, 0.078 v. The familiar kind of curve for loss of photolytic activity (*5*) was thus obtained. Extrapolation of such data shows that at the time of their isolation (zero time) the chloroplasts must be capable of a reducing potential in the negative voltage range.

In other experiments the potentiometric method (to be described elsewhere) was used to determine the course of reduction of several different oxidants by illuminated chloroplasts. The oxidants tested were 2,6-dichlorophenol indophenol, *o*-cresol indophenol, 1 naphthol-2-sulfonate indophenol, toluylene blue, thionine, cresyl blue, methylene blue, indigo tetrasulfonate, and indigo disulfonate, in order of decreasing standard potential. Whereas in air the rate and amount of drop of potential were directly related to the standard potential of the oxidant, in rough agreement with Aronoff's data (*6*), no regular relationships were found in the absence of oxygen. Thus the reoxidation of the added oxidant can obscure the redox state of the chloroplastic reductant. The following data were obtained with anaerobic (nitrogen) reaction mixtures containing 0.05 *M* phosphate buffer at pH 6.5, chloroplasts at a concentration of $5\text{–}10 \times 10^{-6}$ *M* chlorophyll, and oxidants at a concentration of 5×10^{-6} *M*, at 15° C and at a light intensity of about 1000 ft-c. MeBl was reduced the most rapidly—e.g., 1.14 *M*/min/*M* chlorophyll—yet it was not completely reduced. In comparison, 2,6-dichlorophenol indophenol, toluylene blue, and indigo tetrasulfonate were reduced at rates of 0.53, 0.53 and 0.02 *M*/min/*M* chlorophyll, respectively. Also, some dyes of both higher and lower standard potentials than MeBl were reduced by fresh chloroplasts to lower potentials than was MeBl (Table 1). The lowest potential measured was approximately – 0.25 v (E_h), and it was achieved with toluylene blue. There is no reason to believe that this is close to the maximum reducing power of chloroplasts *in vitro*, but this value is probably the nearest of the above to the latter. The possibility of an electrode being poisoned was lessened by agreements between two electrodes per reaction mixture, and by reproducible results. The loss of photolytic activity sustained by the chloroplasts in each reaction mixture, during the course of these experiments, was not determined. However, the various oxidants in a given series of experiments (Table 1) were photolytically reduced by the same preparation of chloroplasts in separate vessels, simultaneously.

TABLE 1

COMPUTED STANDARD POTENTIALS ($E_{0'}$) (VOLT) OF A SERIES OF OXIDANTS AND THEIR NORMAL POTENTIALS (E_h) (VOLT) BEFORE AND AFTER REDUCTION BY ILLUMINATED CHLOROPLASTS

Oxidant	2,6-Dichlorophenol indophenol	Toluylene blue	Cresyl blue	Methylene blue	Indigo disulfonate
pH 6.5 $E_{0'}$ 15° C	0.283	0.169	0.116	0.060	– 0.067
Initial E_h	0.435	0.448	0.496	0.471	0.470
E_h after 2.75 hr	0.071	– 0.239	– 0.046	0.011	– 0.009
Potential drop (volt)	0.364	0.687	0.542	0.460	0.479

It can be concluded that characteristics of the oxidant, other than those associated with its standard potential, affect the apparent reducing activity of illuminated chloroplasts. The fact that photosynthesis occurs in the presence of oxygen and other oxidants of higher potential than CO_2 (*4*) may be another

[1] This work was conducted during part of the tenure of a predoctoral research fellowship of the Carnegie Institution of Washington at its Department of Plant Biology at Stanford, under C. S. French, to whom the writer is deeply grateful.

[2] The terminology is used by which the potential of the oxygen electrode is positive in sign.