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THE EFFECT OF THIOCTIC ACID ON THE QUANTUM EFFICIENCY OF THE HILL REACTION IN INTERMITTENT LIGHT*

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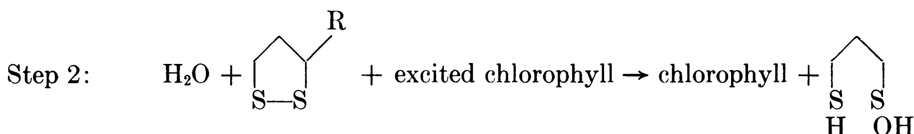
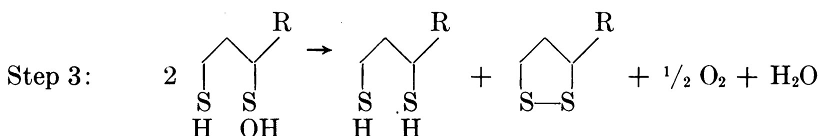
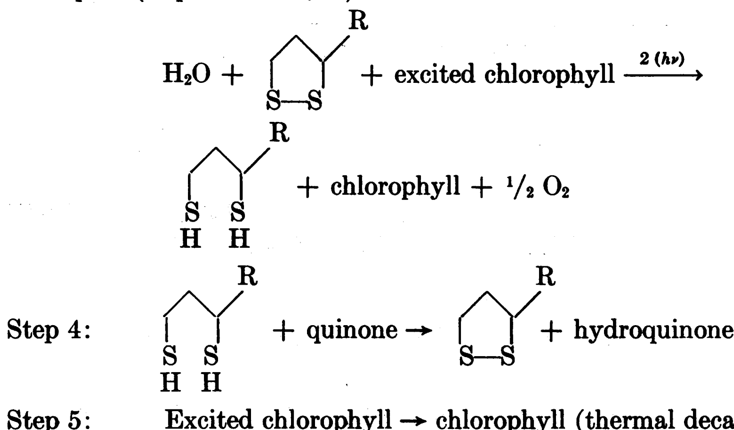
If thioctic acid is an intermediate in photosynthesis closely related to the primary photochemical act as proposed by Calvin and Barltrop,[1] it should be possible to realize experimental conditions under which the concentration of thioctic acid limits the quantum efficiency of photosynthesis as well as the closely related Hill reaction. Conditions under which the amounts of thioctic acid and oxidant, quinone, were simultaneously limiting factors in the Hill reaction in continuous light have been described by Bradley and Calvin.[2] A kinetic model consistent with this simultaneous limitation, the quantum conversion proposal of Calvin and Barltrop, and the photolysis experiments of Barltrop, Hayes, and Calvin[3] has been proposed by Bradley[4] (Model T). This consisted essentially of three steps: (1) a light-capturing step to produce a relatively long-lived electronic excitation; (2) a conversion step in which the "excitation" is converted into some "chemical" form consisting of an oxidizing and a reducing agent; and (3) the reduction of quinone by the reducing agent (in this model, the dithiol of thioctic acid) and the liberation of oxygen from the oxidizing agent. Two sets of more or less specific reactions which conform to these requirements are given below:

Step 1: Light + chlorophyll → excited chlorophyll







Step 5: Excited chlorophyll → chlorophyll (thermal decay)

Model T was shown by Bassham *et al.*[5] to be consistent with the available thermochemical data. The model is more general than that of Calvin[2] or Barltrop[3] or that of Levitt[6] as to the precise mechanism of energy transfer if step 2, and step 2 plus step 3, may be replaced by the less specific step 23.

It should be noted that, while the experiments of Bradley and Calvin are consistent with the quantum conversion process as conceived of by Calvin and Barltrop and by Levitt, the experiments could be carried out only under conditions in which the rate of oxygen evolution was limited by step 1 and/or step 4 and therefore provided no information as to the nature of step 2 (or step 23). Furthermore, these experiments did not establish unequivocally whether thioctic acid has *any* natural, in vivo function in the photochemical processes of photosynthesis.

The pioneering experiments of Emerson and Arnold,[7] in which they carried out photosynthesis in flashing light of high intensity with relatively long dark intervals between flashes, were designed to eliminate the rate limitation of both light capture (step 1) and dark, follow reactions (step 3 and further), the so-called "Blackman reactions" (e.g., steps 3 and 4). The limiting factor then supposedly becomes, in the photosynthetic unit models of Emerson and Arnold and of Wohl[8] (Model PU), the concentration of centers associated with several thousand chlorophyll molecules in the photosynthetic unit which may reduce CO_2 or, in the three-enzyme model of Franck and Herzfeld[9] (Model 3E), the concentration of the enzyme which reacts with the unstable photoproducts, the stabilizing enzyme B. In Model T the corresponding limiting chemical factor would become (in step 2) the concentration of thioctic acid in the disulfide form, provided that no other purely physical transformations which might become limiting were interposed between steps 1 and 2, i.e., the "excited chlorophyll" produced in step 1 is identical with the "excited chlorophyll" used in step 2 or 23.

An important assumption underlying such reasoning is that, when the rate of photon capture exceeds the possible rate of conversion into a suitable stable form (step 2), the excess quanta are lost through back reactions (e.g., step 5). This is based on the further assumption that the back reactions from the excited chlorophyll (step 5) occur rapidly with respect to the time needed to carry out the dark, follow reactions (e.g., steps 3 and 4), which seemed reasonable, since the lifetime

of the metastable state of chlorophyll in vitro has been shown by Livingston[10] to lie between 10^{-3} and 10^{-4} second, while the time for the completion of the dark reactions[7] has been found to lie between 10^{-1} and 10^{-2} second.

We have extended the original experiments of Bradley and Calvin, using the flashing-light technique of Emerson and Arnold in an attempt to eliminate the limitation of oxygen production by the dark chemical reactions, particularly step 4 (the reduction of quinone by thioctic dithiol), so that some information about the nature and possible thioctic acid requirement of step 2 (or step 23) might be obtained.

Experimental.—The experiments were carried out according to the basic protocol described previously[2] in an N_2 atmosphere to eliminate the necessity of a respiratory correction. Each vessel contained sufficient *Scenedesmus* to give an optical density of the extracted pigments from 1-ml. suspension in 10 ml. ethanol at the red maximum of chlorophyll a of 0.8 in a 1-cm. cell. This corresponds nearly to 10 mm^3 *Scenedesmus*/ml, or 20 mm^3 per vessel. Using the value of 8.3×10^4 as the molar extinction of chlorophyll a, this corresponds to 1.9×10^{-7} mole of chlorophyll a per vessel.[11]

Light flashes were produced by discharging capacitors through a GE FT422 high-pressure xenon blue-white flash tube (color temperature, 7,000° K.). The discharge was triggered by a variable high-voltage tickler trigger at rates adjustable from 0.5 to 100 flashes per second. The decay of light intensity was approximately exponential, with a time for half-decay of 160×10^{-6} second. The manometer vessels were so arranged with respect to the lamp that the intensity at 10 microfarads, 2,000 volts, and 10 flashes per second was 8×10^3 ergs/cm^2/sec, corresponding to 8×10^2 ergs/cm^2/flash. The flash rate was measured with a Techtronic scope at high flash rates and manually at rates below 5 per second. The intensity per flash was independent of flash rate in the region used, 5–20 cps. Yields of oxygen per flash were calculated from observed flash rates and the total yield over a 30–60-minute period of illumination.

Experimental Results.—A representative set of experimental values measured on aliquots of *Scenedesmus* from a single harvest is shown in Table 1. The probable experimental error (67 per cent standard deviation) in making such measurements was found to be 0.20×10^{-12} mole O_2/mm^3 *Scenedesmus* for aliquots from the same harvest, with considerably greater variation between harvests of algae on different days.

TABLE 1

DEPENDENCE OF YIELD PER FLASH UPON DARK INTERVAL BETWEEN FLASHES*

(Moles O_2 Evolved/Flash/Mm^3 *Scenedesmus* $\times 10^{12}$)

Dark Interval (Sec.)	Control	With Added 6T (0.27 Mg. or 7×10^{-4} *M*)	With 0.42 × Light	With 0.5 × Quinone
0.05	1.15	2.31	1.08	0.59
0.10	2.25	2.90	Omitted	1.26
0.20	2.96	2.72	1.62	2.70

* Temperature, 15.7° C.; voltage, 1,800 (20 cps.), 1,900 (10), 2,000 (5); *Scenedesmus*/vessel, 22 mm.^{3}; control quinone concentration, 0.0030 *M*; chlorophyll a/vessel, 1.9×10^{-7} mole; capacitance, 6.5 microfarads.

An experiment was carried out to compare the highest yields per flash obtainable with the amount of thioctic acid present in the plant. We are indebted to Dr. R. C. Fuller, who assayed the 6T for us by extracting aliquots of *Scenedesmus* with 6 *N*

HCl for one hour at 120° C., using the *S. faecalis* growth-assay method calibrated against synthetic *dl*-thioctic acid. Flashing-light experiments were carried out on aliquots from the same sample of *Scenedesmus*, the results appearing in Table 2.

TABLE 2

COMPARISON OF YIELD PER FLASH WITH IN VIVO THIOCTIC ACID CONCENTRATION*

(All Values in Moles/Mm³ *Scenedesmus* × 10^{12})

Oxygen per Flash (Duplicate)			6-Thioctic Acid (Triplicate)
Control	With 0.42 × Light	With 2.0 × Quinone	
2.94	1.33	2.32	3.8 ± 1 (mean dev.)

* Temperature, 15.7° C.; voltage, 2,100; *Scenedesmus*/vessel, 25 mm.³; control quinone concentration, 0.0030 *M; Chlorophyll a*/vessel, 1.9 × 10^{-7} mole; flash rate, 5 per second; capacitance, 10 microfarads; dark interval, 0.2 second.

Discussion.—From Table 1 we see that, when the interval between flashes is small, corresponding to continuous light, the oxygen evolution is limited by both quinone and 6T concentrations. These limitations are removed and replaced by light limitation when the dark period is lengthened, in agreement with the prediction based on Model T.

From Table 2 we see that the highest yields in molecules of oxygen per flash, when the dark time is sufficiently long, turn out to be very nearly equal to the total number of molecules of thioctic acid within the same amount of plant material. If we retain the assumption that each molecule of thioctic acid has time to convert quanta only once during the lifetime of the excitation produced by each flash, we arrive at the untenable result that not only does each quantum produce a molecule of oxygen but only one molecule of thioctic acid is involved in that production. We must therefore abandon the original assumption and permit each thioctic acid molecule to go through several complete cycles between successive flashes. Such multiple cycling of thioctic acid would be possible only if nonproductive decay of photoexcitation (e.g., step 5) were relatively slow. We can estimate the minimum lifetime of this excitation by calculating the minimum dark interval required for yield saturation for the four columns in Table 1: control, 2.96/2.25 × 0.1 = 0.13; added 6T, 2.96/2.31 × 0.05 = 0.06; 0.42 × light, 1.62/1.08 × 0.05 = 0.08; 0.50 × quinone, 2.96/1.25 × 0.1 = 0.23 second. Since the yield per flash is the same within probable error whether the recycling process requires 0.06 or 0.23 second for completion, it is evident that no appreciable nonproductive decay of photoexcitation occurs in 0.23 second.

This conclusion at first seems inconsistent with the finding of Livingston[10] that the lifetime of the photoexcited metastable state of chlorophyll in vitro lies between 10^{-3} and 10^{-4} second. However, Witt[12] has recently measured the lifetime of a photoexcited metastable absorption band at 5150 A in vivo in *Chlorella* which is of the order of 0.02 second. The fact that the long-lived photoexcited state of chlorophyll in vitro appears to have enhanced absorption at 5100 A led Witt to identify his excitation with that observed by Livingston. Although species differences might reasonably produce a lifetime as long as 0.06 second for Witt's metastable state in *Scenedesmus*, it is unlikely that his state can be identified with one that exhibits no appreciable decay in 0.23 second. We therefore conclude that in *Scenedesmus* also the metastable state showing enhanced absorption at 5150 A (Witt) decays in 0.02–0.06 second to a very long-lived state, >0.23 second.

The 0.23 second required for dark-time saturation with 0.50 × quinone (0.0015 *M*) is much longer than the time generally reported for photosynthesis and the Hill reaction at or near light saturation, i.e., 0.02 second for photosynthesis in *Chlorella* (Emerson and Arnold[7]) and 0.04 second for the Hill reaction (with quinone) in *Chlorella* (Clendenning and Ehrmantraut[11]). This long time results from quinone concentration limitation, as can be seen from the fact that the dark time for saturation is only 0.13 second at 0.003 *M* quinone. This inverse first-order dependence of minimum dark interval for flash saturation on quinone concentration does not extend far above 0.003 *M* because of the introduction of inhibitory side reactions by quinone. Our dark time for dark-interval saturation is therefore longer than previously observed because the *Scenedesmus* used are the first reported to be quinone-limited. We have grown *Scenedesmus*, for two years in continuous culture, which are quite reproducibly quinone-limited, although *Chlorella* grown under precisely the same environmental conditions are reproducibly non-quinone-limited. It is to be noted that, when quinone limitation is removed by the addition of thioctic acid (Table 1), the dark interval for saturation becomes 0.06 second, in approximate agreement with other values in the literature. The effects of quinone limitation closely parallel those of cyanide inhibition, studied by Weller and Franck,[13] in which the yield per flash is limited at short but not at long dark intervals and the minimum dark time for maximum yield per flash is much longer in the presence of cyanide, i.e., about 0.14 second. Weller and Franck, as well as Rieke and Gaffron,[14] interpret this as inhibition of the enzyme system which speeds the recovery of Catalyst B, and not inhibition of Catalyst B itself.

Speaking in more general terms, without reference to any specific model of the Hill system, it seems quite clear that low quinone concentrations limit oxygen production in continuous and rapid flashing light by limiting the rate at which the dark reactions are able to carry out the necessary oxidation reactions to remove the photoproducts and prepare the system for further photochemical reactions. The minimum dark time at 0.0015 *M* quinone is about 0.23 second; at 0.003 *M* quinone it has been shortened to 0.13 second, and it would be shortened still more by further additions of quinone if the higher quinone concentrations did not have some side effects which destroy the Hill reaction ability. The addition of relatively small amounts of thioctic acid (0.007 *M*) can shorten the minimum dark time to what appears to be its smallest value, i.e., 0.06 second. This thioctic acid effect is possible only with *Scenedesmus*, since it is only in *Scenedesmus* that the slower dark reactions leading to quinone reduction appear to be rate-limiting. A similar phenomenon can be achieved in other algae by partially slowing down some of the dark reactions with cyanide. We are thus led to the notion that, at least in *Scenedesmus*, it is possible to find conditions for the Hill reaction with quinone in which either one of two successive steps might be rate-limiting. The longer (later) one involves the reduction of quinone. When this rate limitation is removed either by raising the quinone concentration or by providing a more efficient hydrogen acceptor and carrier, such as thioctic acid, we then see the smallest time constant as rate-limiting (0.03–0.06 second).

This same time constant, or a corresponding one, has now been observed in three different types of experiments and is likely to be found in a fourth. These are, first, (*a*) the Emerson-Arnold dark times, first observed directly on photosynthetic

oxygen production and carbon dioxide reduction,[7] and (*b*) the corresponding dark times for the Hill reaction on whole *Chlorella*, reported by Clendenning and Ehrmantraut;[11] second, the decay time on whole *Chlorella* of some sort of excitation observed by Witt;[12] and, third, the minimum dark time for oxygen production in the Hill reaction in the presence of thioctic acid (present work). Fourth, an examination of the luminescence observations of Arnold and Strehler[15] indicates very clearly that the luminescence observed therein in all probability consists of at least two different processes. This is to be seen from the fact that (*a*) the apparent order of the luminescence decay changes with temperature, having an appearance of more nearly second order at 6° C. and more nearly first order at 25° C.; and (*b*) the temperature coefficients of the luminescence observed at ~0.1 second after the cessation of illumination vary greatly, being extremely small in the range of 5° and 10° C. and rising to somewhere between 10 and 20 kcal. in the range above 25° C. We thus suppose that this luminescence consists of at least two processes—a slower, high-temperature process, with a relatively high temperature coefficient (activation energy, approximately 15 kcal.), and a very fast process with a very small temperature coefficient. We suspect that this last-mentioned luminescence with the small temperature coefficient arises from the same species that has the 0.02-second decay time observed by Witt and whose transformation is responsible for the shortest minimum dark times measured by Emerson and Arnold (0.02–0.04 second) and by ourselves (0.06 second). The high-temperature luminescence with the long time constant would correspond to the decay of the long-lived excitation observed in our flashing-light experiments.

We are thus brought to the conclusion that the thioctic acid is not likely to be functioning prior to the 0.02–0.06-second transformation but that, after the 0.02–0.06-second transformation has taken place, the products formed by it have a very long intrinsic lifetime, as indicated by the possibility of finding conditions in which the minimum dark time is longer, as, for example, cyanide poisoning or the Hill reaction of *Scenedesmus* with quinone, and that the thioctic acid reacts with the products of this first transformation. A set of reactions which would correspond to such a proposal and which is, in effect, a modified Model T is as follows, and the relationships between the reactions are given in the following chart:

Step 1: Light + chlorophyll ⇌ first-excited-state chlorophyll

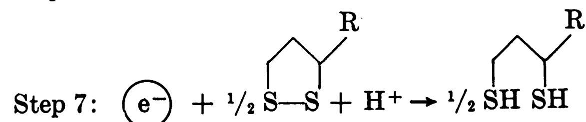
Step 2: First-excited-state chlorophyll → metastable-state chlorophyll

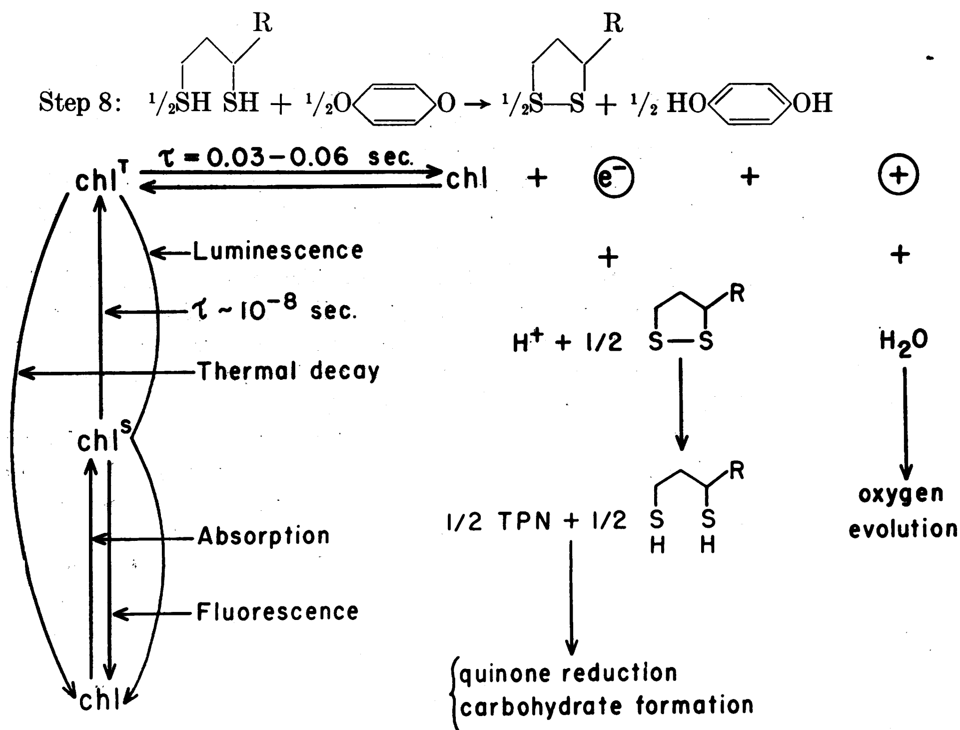
Step 3: Metastable chlorophyll → ground-state chlorophyll + heat

Step 4: Metastable chlorophyll → ground-state chlorophyll + luminescence

Step 5: Metastable chlorophyll ⇌ ground-state chlorophyll + ⓔ⁻ + ⊕

Step 6: ⊕ + H_2O → oxygen evolution





The primary question is, of course, first, the identity of the excitation which has a lifetime of 0.02–0.06 second and, second, the process by which it disappears and the products which are formed as a result of its disappearance. The long-lived (10^{-4} second) excited state of chlorophyll formed in solution (triplet state) has some indication of increased absorption in the range of 5100 A.[16] This has led Witt to identify his excitation, with its 0.02-second lifetime, with the triplet state as observed by Livingston in solution.

While it is possible that the hundred-fold increase in the lifetime of the metastable triplet state in vivo is associated with the rigidity with which the chlorophyll molecule is held in the grana, it seems more likely that the phenomenon is associated with the spectral shift observed in living systems which has been attributed to an interaction between chlorophyll molecules themselves as well as with other species. This leads directly to the concept of a quasi-crystalline lattice in which chlorophyll may actually exist in the plastid.[17] The recent elegant electron micrographs of Steinmann and Sjostrand[18] indicated clearly the presence of rather small, organized structures consisting of flat cylinders about 120 A thick and 3000–5000 A in diameter (and defined by lipid boundaries). The core of the cylinder is presumed to be filled with an aqueous phase, containing the porphyrin part of the chlorophyll molecule in some very definitely ordered array. The photochemical behavior of chlorophyll in such a situation would thus be on the border line between the behavior of molecules randomly oriented in true solution and the behavior of molecules completely oriented in a macrocrystal.[19]

It would appear that the absorption of light could, in such a system, lead either directly or very nearly directly to the formation of conduction electrons and their corresponding holes (ion pairs). These are conceived as being trapped, then, at suitable centers (for example, iron or copper atoms), arranged around or near the surface of the cylinder, where they may await the proper chemicals to take them off. The electron, then, would correspond to the "active hydrogen" and the hole to the "active oxygen" which have been presumed to be formed through the photolysis of water by light.

Since, in all the experiments done heretofore, no evidence has been found of a limitation in the rate of either photosynthesis or the Hill reaction of any step which could be placed along the chain leading to molecular oxygen, we would presume that the hole is immediately trapped, or neutralized, by donation of electrons from a water molecule. This would give the remaining conduction electrons a relatively long life, and we suppose that it is the transfer of these electrons to their primary acceptors which could then become rate-limiting. Thus at least part of the long luminescence observed by Strehler would be controlled by the rate of reaction of these electrons with their primary acceptors and successive reactions. Any oxidizing agent which could accept these electrons should thus obliterate the long luminescence. Further, the long minimum dark time which we have observed in the Hill reaction of *Scenedesmus* with quinone is due in part to this limitation. When thioctic acid is added, this limitation of electron oxidation is removed, and we then see the rate of conduction electron formation (0.02–0.06 second) as the limiting step.

Such a reaction, conceived as a process of diffusion of an "exciton"[20] through a quasi-crystalline lattice until "collision" with a suitable lattice imperfection leads to its conversion by ionization into a conduction electron and a positive hole, would be expected to have a small temperature coefficient and to be dependent upon a certain minimum-size particle.[18] The extremely high efficiency of thioctic acid as an electron acceptor, in spite of its high reduction potential ($\sim$ 0.3 volt), is additional support for the suggestion that this is a natural function.

Summary.—The yield of oxygen per flash has been measured in *Scenedesmus* in flashing light. At dark intervals between flashes of 0.05 second the yield per flash is limited by the concentrations of both quinone and thioctic acid in the medium. When the dark interval is lengthened to 0.2 second, these limitations disappear and are replaced by light limitation. The yield per flash under these latter conditions is nearly equal to the total amount of thioctic acid in the plant on a molar basis, which is consistent with the proposal that thioctic acid is participating in the quantum conversion process in photosynthesis, provided that the lifetime for thermal decay of the photochemically active state (of chlorophyll) is at least of the order of 0.2 second in vivo. This, together with existing data, leads to the suggestion of two distinct physical stages in the conversion of the photon energy into chemical potential, only the second of which may involve thioctic acid.

We wish to express our appreciation to Dr. Wilson Powell and Mr. James Shand of the Radiation Laboratory for designing and constructing our flashing-light apparatus.

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*MAGNESIUM ANTAGONISM OF THE UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY IODO-THYRONINES**

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In parallel with the uncoupling of phosphorylation and respiration by nitro- and halophenols, uncoupling through the natural halophenols, thyroxine, triiodothyronine, and similar analogues has now been shown with increasing precision. Nevertheless, the fact that compounds of the nitrophenol type cannot replace thyroxine physiologically presents difficulty in the extension of such an uncoupling effect toward a generalized interpretation of thyroid hormone action. In the course of work on this problem, we became aware of a curious antagonism of magnesium against uncoupling with thyroxine. This is of particular interest inasmuch as this antagonism appears to be quite specific for the physiologically active iodothyro-