THE UNCOUPLING OF OXIDATIVE PHOSPHORYLATION AS A MECHANISM OF DRUG ACTION[1]

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In the normal metabolism of the cell, oxidation leads to the synthesis of energy-rich phosphate bonds. In the past several years, there has been considerable interest in substances, including drugs, which depress high-energy phosphate bond formation without affecting oxygen consumption. This dissociation of oxidation and phosphorylation is termed "uncoupling". This review will be concerned with uncoupling and uncoupling agents and their possible relation to drug action.

I. THE ROLE OF ENZYMES IN DRUG ACTION

A. General Considerations

Elucidation of the mechanisms by which drugs produce their pharmacological effects is one of the central problems facing the pharmacologist today. It has been suggested in the past (37, 165) that the fundamental action of a drug might be explained by its ability to interact with, or in some way influence the normal function of an enzyme or enzyme system. The fact that many drugs act in minute amounts makes it attractive to speculate that they act on enzymes which are essential in normal cellular function in very small amounts (71). Such functions as growth, reproduction, muscle contraction and nerve conduction involve the integration of chemical reactions catalyzed by enzymes. Therefore, any drug or agent that influences an enzyme or enzyme system directly or indirectly may be expected to have a profound effect upon the behavior of the cell, tissue or organism.

There are few instances in which an action of a drug *in vivo* has been linked unequivocally to an action of the agent on an enzyme system *in vitro*. Englehart and Loewi in 1930 (56) demonstrated that physostigmine exerts its pharmacological action by inhibiting the enzyme cholinesterase. It is also probable that most of the other anti-cholinesterases owe much of their pharmacological activity to their ability to inhibit this enzyme, although this is probably not the only site at which these agents act. Other compounds, such as cyanide, azide and related agents, are toxic to the organism probably because they inhibit the action of certain essential enzymes, the cytochromes.

In any consideration of the mechanism of action of drugs, one should bear in mind that most drugs probably act at more than one site. This author thinks it improbable for a compound to be so highly selective as to act on only one enzyme.

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The possibility exists, however, that one site might be sufficiently more sensitive than another to account for the major actions of an agent *in vivo*.

B. Criteria to be Considered in Evaluating in vitro Effects

Although a drug might inhibit a particular enzyme system *in vitro*, there is still no certain means of ascertaining that the effect observed *in vitro* is related to the known pharmacological actions of the drug. Welch and Bueding (229) suggested that among the criteria to be satisfied before the action of a drug may be attributed to its effect on an enzyme system are the following: (a) The concentrations of the drug or agent which produce the effect on some enzyme system *in vitro* should be comparable to those producing a given pharmacological effect *in vivo*. The concentrations should be similar to those obtaining at the anatomical site of action, *e.g.*, in brain tissue if the drug is known to be a central nervous system depressant. If there is a significant discrepancy between *in vivo* and *in vitro* concentrations it must be shown that there is a selective concentrating of the agent at the site of action. (b) If the drug exerts its effect primarily on a specific tissue *in vivo*, the effect on this tissue *in vitro* should be more pronounced than on other tissues, or the enzyme system inhibited should be shown to have more functional significance in this tissue than in other tissues. (c) Among structurally related compounds there should be close parallelism between pharmacological action and enzyme inhibition. All drugs of the same chemical series which are active *in vivo* must also be active *in vitro*, and those inactive *in vivo* must be inactive *in vitro*, unless the discrepancy can be accounted for by poor absorption, by inadequate distribution to, or penetration into the cells involved, by too rapid excretion or by metabolic alteration.

II. THE PHENOMENON OF UNCOUPLING

The concept that certain drugs may interfere with phosphorylation (or dissociate phosphorylation from oxidation) is not new (120, 151). It is apparent that any interference with phosphorylation and the production of energy-rich compounds would alter the normal function of the cell. A considerable number of drugs or substances which apparently have in common the ability to depress energy-yielding reactions without significantly depressing the normal oxygen consumption of the cell have been reported in the recent literature. Such compounds have been termed "uncoupling" agents because they are capable of disrupting or uncoupling the link between oxidation and phosphorylation, thus depriving the cell or organism of its useful energy. Other compounds exert their primary effect on oxidation and the inhibition of phosphorylation parallels the reduction in respiration. Uncoupling always implies that the phosphorylative mechanisms are more sensitive to the action of an agent than are the oxidative steps.

A. The Role of Oxidative Phosphorylation in Cell Metabolism

In the energy metabolism of cells, tissues and organism, the consumption of oxygen and the production of high-energy phosphate compounds are interdepend-

ent phenomena. This concept of phosphorylation "coupled" to oxidation has developed gradually since 1930.

The observation that pyrophosphate compounds are generated by pigeon red blood cells during the consumption of oxygen was made by Engelhardt (55). Confirmation of this work was reported by Lennerstrand and Runnström (134) and by Kalckar (104) who observed a respiration-dependent conversion of inorganic phosphate to hexose diphosphate in kidney extracts. The latter worker extended his studies (105) to show that the oxygen consumption of kidney extracts was increased by the presence of such substrates as citrate, glutamate, fumarate or malate and that simultaneously adenylic acid, glucose or glycerol was phosphorylated. Belitzer and Tsibakova (19) and Colowick *et al.* (42) subsequently confirmed the original studies of coupled oxidation and phosphorylation.

The efficiency of the phosphorylating mechanism is usually expressed as the phosphate-to-oxygen ratio, *i.e.*, the ratio of the moles of inorganic phosphate esterified per atom of oxygen consumed. Belitzer and Tsibakova (19) and Ochoa (167) obtained phosphate-to-oxygen (P:O) ratios greater than 1.0, indicating that more than one energy-rich phosphate bond was generated per atom of oxygen consumed. Ochoa (168) proposed that 3.0 should be the average P:O ratio for the oxidation of α-ketoglutarate, although his experiments yielded ratios somewhat less than this value. Recent studies have established P:O ratios of 3.0 and above (18, 43, 94, 115), confirming Ochoa's prediction.

It is well-known that the oxidation of a Krebs-cycle intermediate (*e.g.*, succinate) involves a chain of reactions. By means of flavoproteins, cytochromes, etc., hydrogen and electrons are transferred from substrate to atmospheric oxygen. Apparently, high-energy phosphate bonds are formed not only at the initial step, but also at several other points along the chain of reactions. Attempts have been made to show inorganic phosphate uptake during these later steps of the electron transport chain (61, 132, 205). The number of energy-producing steps in this chain seems to vary for the several Krebs substrates (96, 115, 206). The observed P:O ratios differ for the different substrates. For example, the oxidation of succinate consistently yields ratios around 2.0 whereas with pyruvate the ratio is approximately 3.0. It has been generally accepted that the P:O ratio or net uptake of inorganic phosphate is an accurate measure of the ability of the tissue to synthesize high-energy bond compounds in an *in vitro* system.

B. Methods of Studying Uncoupling

There are a number of ways to demonstrate the ability of a drug to dissociate oxidation and phosphorylation. These include the use of the intact animal as well as such *in vitro* systems as the tissue slice, the tissue homogenate and the isolated mitochondrial preparation.

1. The intact animal. A number of observations have been made in the intact animal which are presumed to relate to the uncoupling action of agents such as dinitrophenol or dinitrocresol. The injection into animals of these compounds decreases phosphocreatine and adenosine triphosphate levels and increases inorganic phosphate levels in most tissues. The very rapid induction of rigor mortis by these

agents has also been associated with this depletion of energy-rich compounds. The treated animals may also show an increased oxygen consumption and a fall in liver glycogen.

Hyperthermia is another presumed result of decreased phosphorylative and increased oxidative capacity produced by uncoupling agents. It is postulated that in treated animals, the energy produced is not converted to bond-energy but rather is dissipated as heat. This hyperpyrexia has been demonstrated with a large number of agents chemically related to the phenols.

At best, the intact animal is a rather unsatisfactory preparation for studying uncoupling action. The above observations may merely be the net result of drug action at several sites. Nucleotide levels, for example, are in reality the balance between the generation and utilization of these compounds. The effect of uncoupling agents on nucleotide utilization is not known. Temperature regulation is also known to be a complex phenomenon. Furthermore, many drugs which are not uncoupling agents may produce one or more of the above effects.

2. The tissue slice or intact cell. The use of the tissue slice or intact cell as a means of determining uncoupling action is rather limited. Thin slices of tissue are cut, placed in a suitable medium, oxygenated and the oxidation of substrate by the slice is measured using the conventional manometric method. The addition of low concentrations of an uncoupling agent will stimulate the oxygen consumption of such a preparation. Because of the numerous simultaneous reactions occurring within the slice, quantitative data (P:O ratios) regarding the efficiency of oxidative phosphorylation cannot readily be obtained.

While low concentrations of uncoupling agent stimulate oxygen consumption, it is usually depressed when the concentration of the agent is increased. This is probably a direct inhibition of the oxidative enzymes by these compounds. While most uncoupling agents do stimulate the respiration of intact cells *in vitro* and this response is often used as a means of confirming uncoupling action, several uncouplers, *e.g.*, chlortetracycline and thyroxin, fail to increase oxygen consumption in such a preparation. Furthermore, certain drugs which increase the respiration of tissue slices fail to dissociate phosphorylation from oxidation when tested in a more specific system. Such is the case with the cardiac glycosides which have been shown to stimulate the oxygen consumption of cardiac muscle slices at low drug concentrations. Ouabain fails to uncouple phosphorylation when assayed in a heart muscle mitochondrial preparation (119).

3. The tissue homogenate. This cell-free preparation is prepared by homogenizing the tissue in a suitable isotonic medium. It is usually fortified with cofactors and coenzymes since there is considerable dilution during homogenization. The enzymes in this preparation are dissolved in, or suspended in the reaction medium. Thus, it differs from the tissue slice where cell barriers separate the oxidative enzymes from the medium. Because the P:O ratios can be obtained with this preparation, it lends itself to the study of oxidative phosphorylation and uncoupling agents. Oxygen consumption can be measured manometrically and high-energy phosphate bond synthesis can be determined by the disappearance of inorganic phosphate from the reaction medium. The homogenate preparation

has some disadvantages. It contains glycolytic enzymes and other enzymes which result in inaccurate quantitative values for oxygen and phosphorus. The tissue homogenate has no advantage over the isolated mitochondrial preparation.

4. The mitochondrial preparation. The development of subcellular tissue preparations was responsible in a large measure for the recent advances in the field of oxidative phosphorylation. Early observations indicated that certain enzymes were localized in the soluble fraction of the cell, while others were in the insoluble fraction. Warburg (225) showed that the ability of a tissue to take up oxygen resided almost completely in the insoluble particulate fraction. It is now known that the particulate fraction is made up of a large number of enzymes concerned with the oxidation of citric acid-cycle intermediates (74), the oxidation of fatty acids (130), electron transport (196, 198) and numerous other reactions (72, 199). It has also been established that the enzymes which synthesize the high-energy phosphate compounds during aerobic oxidations are associated with the insoluble fraction.

Further separation of the insoluble particles by anatomists Bensley and Hoerr (20) using the technique of differential centrifugation led the way to the biochemical studies of the various cell fractions. Green *et al.* (74) employing a heterogeneous washed kidney residue which was termed the "cyclophorase system" demonstrated that all of the Krebs cycle intermediates could be burned to carbon dioxide and water by this preparation. It was found subsequently that the so-called "cyclophorase" activity was contained within the mitochondrial fraction of the cell (80, 107, 108, 201). The significant studies in the separation of cell fractions was done by Hogeboom *et al.* (89) who utilized sucrose, a non-electrolyte, as a dispersing agent in the preparation of tissue homogenates. This work eventually led to methods whereby relatively large amounts of nuclei, mitochondria and microsomes, sufficient for biochemical reactions, could be isolated. A large number of enzymes in addition to those mentioned have been associated with the mitochondrion; for further details on this subject the reader is referred to the reviews of Green (73), Lehninger (133), Schneider (195) and Potter *et al.* (181).

Mitochondria isolated from a variety of sources appear to possess identical biochemical properties. The original studies were carried out on mitochondria from rat and rabbit liver and kidney. However, mitochondria from heart muscle (38, 80, 180), striated muscle (109) and spleen (8) seem to contain the same enzyme complement. Present data indicate that mitochondria from various species show few differences. The particulate fraction has also been of considerable interest in the study of tumor tissue metabolism (88, 197, 200, 236). In addition to the unique biochemical properties exhibited by mitochondria, they are known to contain certain biologically potent materials. Especially interesting to the pharmacologist are the findings that epinephrine (21), histamine (44), cholinesterase (240) and amine oxidase (45, 81) are largely contained in this cellular fraction.

Recent studies have demonstrated that mitochondria can be isolated from the brain (29) and spinal cord (2) by the method of differential centrifugation. These granules have properties similar to those of mitochondria from other tissues al-

though a few differences have been noted (29). These enzymatically active particles are no doubt contaminated to some extent by other intracellular fractions. However, when studying drugs whose primary action is on the central nervous system, a brain mitochondrial preparation would seem to have more validity than a similar preparation from other tissues.

As has been mentioned, the currently-popular methods of determining whether or not a drug is an uncoupling agent make use of the mitochondrial preparation.

a. The hexokinase trap. One of these methods utilizes the "hexokinase trap" as described by Cross *et al.* (46). In a mitochondrial preparation, the high-energy bonds synthesized during the oxidation of a citric acid cycle intermediate are trapped as glucose-6-phosphate. Hexokinase, glucose and catalytic amounts of adenosine triphosphate (ATP) are added and the high-energy phosphate bonds formed during oxidative phosphorylation are trapped according to the following reactions:

$$(1)\quad \text{inorganic phosphate} \xrightarrow[\text{oxidations}]{\text{citric acid cycle}} (\sim \text{P})$$

$$(2)\quad (\sim \text{P}) + \text{ADP} \longrightarrow \text{ATP}$$

$$(3)\quad \text{ATP} + \text{glucose} \xrightarrow[\text{hexokinase}]{} \text{glucose-6-phosphate} + \text{ADP}$$

(~P) is the primary energy-rich enzyme or intermediate. There may be other energy-rich compounds formed prior to the final transfer to adenosine diphosphate (ADP), but these have not been indicated in the above scheme. Glycolytic enzymes have been removed during the fractionation of the homogenate so that glucose-6-phosphate is not degraded. Dephosphorylation leaks are minimized by using an ATPase-inhibitor in the system. The uptake of inorganic phosphate or the level of inorganic phosphate remaining in the reaction medium thus serves as an index of the phosphorylating ability of the tissue. From these data and the concomitant oxygen uptake the P:O ratio may be calculated in the presence and absence of the drug under investigation. The effect of the agent on the glucose-hexokinase trap should also be studied, obviously any inhibitory action of the drug on the kinase reaction will result in a depression of the P:O ratio.

b. The phosphate-deficient system. Another method by which the uncoupling potential of an agent can be tested is by the use of the "phosphate-deficient" system (24, 102). It is well known that up to certain levels the concentration of inorganic phosphate in the reaction medium will regulate the rate of oxidation (124, 185). In the phosphate-deficient system the concentration of inorganic phosphate is reduced so that oxidation will proceed at a very slow rate. Under these conditions in the presence of an uncoupling agent, oxygen uptake is stimulated and reaches values ordinarily obtained with optimum phosphate levels. The presence of the uncoupling agent thus mimics the effects of the addition of inorganic phosphate to the medium. However, the uncoupler apparently cannot replace orthophosphate because this result can no longer be obtained if the mito-

chondrial preparation is extensively washed to remove all but traces of inorganic phosphate (218).

c. The acceptor-deficient system. Similar stimulation can be obtained with an uncoupling agent if the reaction mixture is made deficient in acceptor such as ADP (22, 124).

5. Miscellaneous preparations. In addition to the preparations mentioned, any synthetic reaction where phosphate-bond energy is known to be essential may also serve as a test system for a drug suspected of being able to dissociate phosphorylation from oxidation. The fatty acid oxidase system is one commonly employed, although others may be used (106).

III. AGENTS THAT UNCOUPLE PHOSPHORYLATION

The agents that uncouple phosphorylation from oxidation fall into two general types. The more classical type, exemplified by the nitrophenols and the barbiturates, stimulates respiration in phosphate-deficient *in vitro* systems and is not dependent upon magnesium for its action. A second type of uncoupling agent exerts its effect by its interaction with magnesium ion. To this second group belong such substances as tetracycline antibiotics and thyroxin. These substances produce *in vitro* uncoupling only when the drug is incubated in a medium which is low in magnesium ion content. The addition of an excess of magnesium will prevent or reverse the inhibition of phosphorylation. In addition, these latter drugs will not stimulate the respiration of a phosphate-deficient or acceptor-deficient system.

A. Hypnotic Drugs

The interaction of hypnotic drugs with enzymes has been subjected to numerous studies. Details of the theories of metabolic inhibition by anesthetic and hypnotic agents may be obtained from the excellent review of Butler (35). It has been postulated that central nervous system depressants in general produce their effects by inhibiting oxidative reactions in the central nervous system, the pioneer work in this area being largely that of Quastel and his associates (100, 101, 182, 183, 184). A considerable number of drugs have been studied and it has been demonstrated that anesthetics and hypnotics are not unique in their depression of respiration of brain tissue *in vitro*. A number of other agents, including stimulants, produce a comparable inhibition (203).

1. The barbiturates. a. In vitro studies. The derivatives of barbituric acid have been studied extensively and attempts have been made to correlate the depressant properties of these agents *in vivo* with their inhibitory action on tissue respiration *in vitro* (62). A number of important difficulties in attempting to relate the *in vivo* activity to the *in vitro* effects have been discussed in detail by Bain (11). There has also been considerable interest in the precise locus of the barbiturate block. Michaelis and Quastel (161) and Greig (75) indicated that the flavoprotein step in the hydrogen transport system might be the depressant-sensitive area. Persky *et al.* (176) have demonstrated that high concentrations of barbiturates blocked the pyruvic acid oxidase system and postulated that the sulfhydryl

groups of the dehydrogenase might be barbiturate-sensitive. This latter hypothesis could not be confirmed by Greig (77). Other partially purified enzyme systems have been studied and each has failed to show any unique sensitivity to the barbiturates.

The chief objection to the studies on oxygen consumption of brain slices or homogenates and those on isolated enzyme systems is that the inhibitory effects were demonstrated with relatively large amounts of drug (1 to 5 $\times$ 10^{-3} M of the potent hypnotics). Drug concentrations of this magnitude would be considerably in excess of the concentration in the brain when anesthesia is produced with these agents. Even the ability of the central nervous system to concentrate the barbiturates several-fold, which has not been demonstrated (67, 91, 111, 224), would not account for this discrepancy in concentrations. It might be expected that if the brain could concentrate these agents *in vivo*, concentration of these drugs should logically be expected to take place *in vitro* from the suspending medium into the brain slice (35). Such is not the case; nor is there any evidence available to indicate that the barbiturates may selectively depress the respiration of a particular area of the brain (232, 233).

There are other objections to the postulate that the inhibition of oxidation *per se* is responsible for the observed pharmacological activity of the barbiturates. These included the observations of Larrabee *et al.* (125, 126) that synaptic transmission can be depressed in the presence of certain anesthetic agents and that this depression of function is independent of any measurable effect on the rate of oxygen consumption. Pentobarbital, for example, at a concentration of 5 $\times$ 10^{-4} M, depressed synaptic transmission by more than 50 per cent with no effect on oxygen uptake. Himwich *et al.* (86) reported that the cerebral metabolic rate in the intact animal was not diminished by a barbiturate until deeper levels of anesthesia were reached. Considerable depression of function could be demonstrated before any inhibition of the metabolic rate was apparent. Westfall (231), using rat brain cortical slices, has shown that low concentrations of barbiturates actually increase the consumption of oxygen *in vitro*. Other objections have been brought forward which would indicate that the inhibition of oxidation *per se* is not a satisfactory explanation for barbiturate action (11).

In 1951, Brody and Bain reported that thiopental, pentobarbital and amobarbital were uncoupling agents (27). This effect was found using rat and rabbit brain particulate preparations and with drug concentrations (1 to 5 $\times$ 10^{-4} M) which were roughly equivalent to levels found in the intact animal under barbiturate anesthesia. The studies were carried out with pyruvate plus fumarate as the substrate and included the yeast hexokinase trapping system. The barbiturates were found to have no inhibitory effect on the hexokinase reaction in drug concentrations as high as 5 $\times$ 10^{-3} M. Subsequent studies by the same workers (30) indicated that this uncoupling phenomenon could be demonstrated with each of the intermediates of the Krebs citric acid cycle for all of the barbiturates investigated. Both liver and brain mitochondria were used as enzyme sources and in each instance the brain preparation proved to be more sensitive to the barbiturate. The uncoupling effect with succinate as substrate is of con-

siderable interest because Quastel and Wheatley (183) had demonstrated that the barbiturates had no inhibitory action on succinate oxidation. As a result of Quastel and Wheatley's finding, this intermediate was used as an antagonist in the treatment of barbiturate depression (208). Thus the failure of other workers (122, 194) to successfully antidote the barbiturates with succinate may be explained.

Johnson and Quastel (98) have claimed that the uncoupling actions of dinitrophenol and the barbiturates are dissimilar. They based this conclusion on the finding that dinitrophenol inhibited, while pentobarbital failed to inhibit the acetylation of sulfanilamide by pigeon liver extracts. However, these workers obtained 50 per cent inhibition of acetylation with 5×10^{-4} M dinitrophenol, a concentration so large that it will depress many functions in addition to uncoupling phosphorylation. Conversely, Govier and Gibbons (70) reported that a high concentration of pentobarbital would inhibit a similar system. These latter workers employed aged bicarbonate extracts of pigeon liver acetone powder in the presence of added coenzyme A, and could reverse the pentobarbital inhibition by additional coenzyme A.

There are a number of striking similarities evident when a barbiturate and dinitrophenol are studied in parallel systems (30). The slopes of the inhibition curves of these compounds are remarkably similar, although dinitrophenol is the more potent agent. Both depress fatty acid oxidation and stimulate the oxidative rate in a phosphate-deficient system.

The addition of excess magnesium ion does not reverse the uncoupling action of either the barbiturate or dinitrophenol.

Some differences between the uncoupling effects of the oxy- and the thiobarbiturates have been reported (30). These have been demonstrated in studies of the reversibility of the uncoupling phenomenon. Uncoupling by the oxybarbiturates and dinitrophenol was reversed by washing when either liver or brain mitochondria were used. Thiopental uncoupling was reversible in liver preparations but not in brain, whereas thioethamyl and thiamylal effects could not be reversed in either tissue preparation. Whether this is due to the persistent attachment of the thiobarbiturate or to some permanent drug-induced change in the mitochondria has not been ascertained.

The findings that barbiturates can uncouple oxidative phosphorylation may explain certain other observations. The work of Westfall (231), mentioned previously, and the studies indicating that low concentrations of barbiturates stimulate glycolysis (226) can now be explained by the levels of inorganic phosphate and adenylate acceptors. The uncoupling agent increases the availability of both of these constituents. The inhibition of acetylcholine synthesis by the barbiturates (160), the failure of succinate to reverse barbiturate depression (122) and the inhibition of ganglionic transmission in the absence of a diminished oxygen consumption (125, 126) may be due in part to the uncoupling action of the barbiturates which prevents the cell from generating high-energy bond compounds and thus depresses function.

b. In vivo studies. One may ask, if the uncoupling action of the barbiturates

in vitro is related to the hypnotic effects of these drugs, why then are not other uncoupling agents hypnotics or anesthetics? Work indicating that a number of uncoupling agents can potentiate barbiturate anesthesia has been reported (32). Mice were pretreated with the uncoupling agent and then challenged with anesthetic doses of secobarbital or amobarbital. The increase in sleeping time was a measure of the potentiating effect. Both dinitrophenol and 2,4-dichlorophenoxyacetate, the latter agent an uncoupler at high concentrations (24), significantly increased the sleeping time. Dinitrophenol, when administered alone, had a slight depressant action, but only if given in doses that approached the lethal level (32).

The inability of the phenolic uncoupling agents to produce hypnosis may be due in part to their poor penetration into the central nervous system. Pentachlorophenol, recently demonstrated to be an uncoupling agent *in vitro* also produces the systemic effects characteristic of dinitrophenol, *i.e.*, hyperthermia and an increased metabolism. Deichmann *et al.* (48) have studied the fate of this compound in the rabbit and other species after the administration of approximately 100 mgm./kgm. It appears that an extremely small amount (less than one-tenth of one per cent) of the administered dose can be recovered from the central nervous system. Inability to enter the brain may account for the lack of central nervous system symptomatology when the nitro- or halophenols are injected into animals, and may be one of the reasons why these uncoupling agents are not depressants.[2]

Another observation indicating potentiation with uncoupling agents is that of Stamps *et al.* (211) who have noticed that when chlortetracycline is combined with phenobarbital in the treatment of epileptic patients, much more sedation is produced than results from the use of the barbiturate alone.

A wide variety of agents has been shown to potentiate barbiturate anesthesia. These include glucose (117), potassium ion (127), epinephrine, lactate and glutamate (118) and various other carbohydrate intermediates (116). The potentiation of barbiturate anesthesia with these latter agents does not seem to be correlated in any way with the uncoupling action of the barbiturates and the mechanism of action of these compounds in potentiating anesthetic action remains unexplained.

Non-hypnotic members of the barbiturate series such as barbituric acid and 5-ethyl barbituric acid (26) fail to uncouple oxidative phosphorylation. However, a convulsant barbiturate, 5-(1,3-dimethylbutyl)-5-ethyl barbituric acid, is a potent uncoupler of phosphorylation (30). Small doses of this agent when injected into animals produce seizures (214), but these are atypical when compared to the seizure patterns exhibited by the classical convulsant drugs (51). Recent work has demonstrated that pretreatment with this convulsant barbiturate will increase the sleeping time in rats challenged with a depressant barbiturate (135). Electroencephalographic studies have shown that threshold convulsive doses (2 mgm./kgm.) have little or no effect on the brain wave pattern of the cat (51). When the dose is increased (6 to 10 mgm./kgm.), electrical activity was definitely

[2] See also Huston, M. J. and Martin, A. W., Arch. internat. pharmacodyn., **101**: 349–357, 1955.

decreased and in several animals an iso-electric cortex was observed. The diminution of cortical activity was not the result of a fall in systemic blood pressure. The higher dose of the convulsant barbiturate was also effective in raising two-fold the seizure threshold to pentylenetetrazole. It might be pointed out here that the convulsants, picrotoxin and pentylenetetrazole, in concentrations as high as 1×10^{-2} M, fail to lower the P:O ratio when assayed in a brain mitochondrial system (26), although these agents do inhibit the oxygen consumption of the brain minces (203).

In the intact animal, the levels of phosphates and nucleotides in the brain are presumed to reflect the functional activity of the central nervous system. It is known that either electrical stimulation of the brain or the injection of a convulsant such as pentylenetetrazol will produce a decrease in ATP and phosphocreatine and an increase in ADP and inorganic phosphate (14, 110, 129, 170, 187, 212). These changes indicate an increase in the utilization of energy-supplying compounds.

The effect of barbiturates upon brain nucleotide levels seems paradoxical and represents the major evidence against the uncoupling hypothesis. If the primary action of the barbiturates is to uncouple phosphorylation from oxidation, one would expect the depressant to cause a depletion of high-energy phosphate compounds and an increase in inorganic phosphate. Generally, the reverse has been found. After the administration of barbiturate the amount of ATP and phosphocreatine was increased and inorganic phosphate decreased (34). Some workers believe that the increased concentrations of high-energy phosphate compounds in the brain are the result of decreased activity (35, 64, 151, 187) and the barbiturates produce their action by inhibiting the utilization of energy. It has been reported, however, that these drugs do not normally influence ATP dephosphorylation activity (11, 143), although under special conditions high barbiturate concentrations will augment the breakdown of ATP (150). This latter action is probably not a significant factor *in vivo*.

Recently, Kozawa *et al.* (111a) demonstrated a reduction of energy-rich phosphate compounds in rabbit brain after the administration of a hypnotic barbiturate. Narcosis was determined by recording electroencephalographically from one hemisphere and the tissue from the other hemisphere was removed for chemical analysis.

It should be emphasized that the concentration of energy-rich compounds in the brain is the net result of the generation and utilization of these compounds. For example, an inhibition of both generation and utilization would result in essentially unchanged levels of high-energy phosphate compounds in the brain.

Attempts to study the uncoupling action of the barbiturates *in vivo* have proven unfruitful. In these experiments anesthetic doses of barbiturate are administered to rats and the animals sacrificed during anesthesia. Removal of the cerebral cortices, isolation of the mitochondria and determination of the P:O ratio indicated no difference in the phosphorylating ability of the barbiturate-treated rats when compared with control animals under the conditions of the experiment (26). These results should probably be expected since the numerous manipulations

involved in the preparation of the mitochondria reduce considerably the concentration of the barbiturate.

c. Is uncoupling related to the depressant action of the barbiturates? Studies with the barbiturates indicate that the uncoupling phenomenon may be responsible for the observed pharmacological action of this class of compounds. Evidence which makes this hypothesis attractive includes the following: 1) inhibition of oxidative phosphorylation is produced *in vitro* by concentrations of drug which obtain *in vivo*; 2) brain tissue is more susceptible to this action than are other tissues; 3) *all* hypnotic barbiturates uncouple phosphorylation and there is a parallelism between *in vivo* effectiveness and *in vitro* uncoupling; 4) barbiturate derivatives which have no depressant action also fail to uncouple oxidative phosphorylation; 5) while other chemically-unrelated compounds which uncouple are not depressants, some may potentiate anesthesia; and 6) a convulsant barbiturate which stimulates the intact animal and is a potent uncoupling agent, may actually depress the cerebral cortex. The prime objections to this hypothesis are 1) the finding that the levels of energy-rich phosphorylated intermediates in the brain are not depressed, but may actually be increased; and 2) the failure of many uncoupling agents to depress the central nervous system.

2. Miscellaneous hypnotics and anesthetics. Other central depressants do not appear to inhibit oxidative phosphorylation. The gaseous anesthetics xenon and nitrous oxide have no effect on the P:O ratio during the oxidation of pyruvate by brain mitochondria (138). However, it has been claimed that ether in concentrations which obtain during anesthesia does depress oxidative phosphorylation (92).

Methadone has been shown by Greig to inhibit the hexokinase reaction (76) although this action of methadone could not be confirmed by Bain (12). This analgesic will stimulate the respiration of brain slices *in vitro* (162) and is also an uncoupling agent in other systems (12), but produces these effects only at concentrations which far exceed the amounts that would be expected to be present in the brain when analgesic doses are administered. Morphine, which is not an uncoupling agent, decreases the phosphocreatine of rat brain and increases lactate, pyruvate, hexose diphosphate and inorganic phosphate (5).

B. Antibiotics as Uncoupling Agents

1. Gramicidin. The earliest observation that an antibiotic may exert its antibacterial effects by uncoupling phosphorylation from oxidation was that of Hotchkiss (90). Gramicidin, a polypeptide, could inhibit growth but increased the bacterial respiration several-fold and decreased the uptake of inorganic phosphate from the medium. It was postulated that gramicidin blocked some energy-utilizing step in the metabolism of the organism. The uncoupling action of gramicidin was confirmed by Cross *et al.* (46) using a cell-free preparation from rabbit kidney. The similarity of the action of this antibiotic to that of dinitrophenol was noted.

2. The tetracycline antibiotics. a. The uncoupling phenomenon. Loomis (140) reported that chlortetracycline was a specific inhibitor of phosphorylation in kidney particulate preparations, whereas it did not affect oxygen consumption.

This observation was verified (28), although oxytetracycline, an antibiotic having similar bacterial spectrum (230) and structure, failed to produce this effect. A further study indicated that chlortetracycline, while depressing the P:O ratio, apparently did not stimulate the oxidative rate of a phosphate-deficient mitochondrial assay system (28). The classical inhibitor, dinitrophenol, will stimulate the respiratory rate several-fold in a phosphate-limited system (102). On the other hand, both chlortetracycline and oxytetracycline are inhibitors of fatty acid oxidation by rat liver mitochondria (31). Van Meter *et al.* (221) had reported that chlortetracycline inhibition of oxidation by rat liver mitochondria could be reversed by the addition of magnesium, especially in aged preparations. The effect of magnesium on the inhibition of oxidative phosphorylation was not specifically mentioned.

Later investigations (31) demonstrated that the uncoupling effects of chlortetracycline could be reversed by the addition of magnesium to the reaction medium. If the concentration of magnesium in the medium was lowered, oxytetracycline and tetracycline could also depress oxidative phosphorylation. The inhibition of fatty acid oxidation by all three of these antibiotics was also dependent upon the magnesium concentration in the medium. Reversal of the inhibition of oxidative phosphorylation could be effected with magnesium although the amount of ion needed to reverse the inhibition varied with the compound. Chlortetracycline was found to be twice as potent an inhibitor of phosphorylation as the other two antibiotics and more magnesium was needed to reverse its action. These results were obtained with both rat liver and brain mitochondria.

It is apparent from the above study that the uncoupling action of the tetracycline antibiotics is brought about in a different manner than that produced by the classical uncoupling agents. However, the overall effect, a depression of phosphorylation, is the same.

There is considerable evidence available which indicates that the action of these antibiotics is dependent upon their ability to chelate or combine with essential cations. Saz and Slie (191, 192, 193) have demonstrated that manganese can reverse the chlortetracycline inhibition of bacterial nitro-reductase, although the effect may not be related to the antimicrobial action of the drug. Compounds chemically related to chlortetracycline yet lacking the antibacterial activity also possess this inhibitory action. Soncin (207) found that aerobic glycolysis, respiration and multiplication of *E. coli* are inhibited by chlortetracycline, oxytetracycline and chloramphenicol and that these effects were readily reversed by magnesium chloride. Weinberg (228) has recently shown that certain multivalent ions can reverse the inhibitory action of oxytetracycline on the growth of sensitive strains of *Pseudomonas aeruginosa*. Magnesium salts appeared to be most effective in reversing the inhibition. An interesting observation is that of Ghatak and Murti (65) who demonstrated that certain antibiotics can depress alkaline phosphatase activity but that magnesium could protect the enzyme against the inhibition produced by oxytetracycline. The above observations bear out the data of Albert (7) who showed that the tetracycline antibiotics have an affinity for metal cations.

There are at least two ways in which the observed effects of the tetracycline antibiotics on oxidative phosphorylation may be explained. 1) The antibiotics react directly with the enzyme or enzymes and thereby produce the inhibitory effect. The reversal by magnesium reflects a removal of the inhibitor by the formation of a slightly dissociated complex. Or 2) these antibiotics inhibit by reacting with magnesium and removing this essential ion from the reaction medium. The present data make the second possibility the more attractive one.

b. The role of uncoupling in the antimicrobial activity. As yet, the evidence that the specific antibacterial action of the tetracycline drugs is due to their ability to inhibit oxidative phosphorylation is not convincing. The concentration of antibiotics (1×10^{-4} M) necessary to depress phosphorylation in the mammalian cell-free preparations is considerably higher than the amount needed to inhibit bacteria.

Miura *et al.* (163) have shown in several bacterial species that oxytetracycline interferes with the incorporation of inorganic phosphate into the organic phosphate fraction and suggest that both chlortetracycline and oxytetracycline have similar mechanisms of antibiotic action. The results reported cannot be translated into "mechanism of action" of the antibiotics unless additional studies are carried out at relevant concentrations and using appropriate bacteria.

c. Uncoupling and host toxicity. The depression of oxidative phosphorylation by the tetracyclines might explain some of their side effects and toxicity. Yesner and Kunkel (239) reported that large doses of these antibiotics could produce fatty changes in the liver, and Lepper *et al.* (136, 137) described microscopic fatty change in the livers of humans receiving chlortetracycline therapy. Liver function was also impaired. Similar gross and histologic changes were produced in dog and mouse liver both with chlortetracycline and oxytetracycline. The results obtained with chloramphenicol were not conclusive. A recent study (202) indicated that the intravenous administration of any of the tetracycline antibiotics increased the hepatic fat content. This increase in liver fat could not be reversed with methionine, methionine and choline, BAL, B vitamins, heparin, penicillin or magnesium sulfate. Heat-inactivated antibiotic did not increase the fat content of the liver.

Certain of the barbiturates (186) are also known to produce fatty changes in the liver.

The mechanism by which the tetracyclines produce hepatic change may be two-fold: 1) the inhibition of oxidative phosphorylation, and 2) a depression of the ability to oxidize fatty acids. Other systems that are magnesium-dependent may also be affected. That the depression of phosphorylation and the liver changes observed may be related seems plausible because comparable concentrations of antibiotics are needed to produce these effects. This finding may have little importance in therapy since the drug concentrations needed to produce fatty livers are so large.

3. Miscellaneous antibacterials. Usnic acid, an antibiotic obtained from a lichen species, also has the property of uncoupling phosphorylation (99) in washed rat liver and kidney particles. Stimulation of oxidation was observed which would

indicate that the uncoupling was of the classical type, and not magnesium-concentration dependent. The concentration of this antibiotic necessary to dissociate phosphorylation from oxidation was comparable to amounts found to inhibit cell growth (147).

Hexachlorophene in low concentrations has the property of uncoupling when tested in tissue mitochondrial preparations (26). This finding probably could be anticipated since hexachlorophene is a halophenol, and many halophenols are uncoupling agents. However, other enzyme systems are also sensitive to hexachlorophene (69) and the present data are not sufficient to decide whether or not the uncoupling phenomenon is important in the bacterial action of this compound.

C. The Salicylates

1. Studies on tissue slices. Several antipyretic agents, including sodium salicylate and acetylsalicylic acid, have been studied on the metabolic activity of brain slices *in vitro* with the hope that such investigations might yield information regarding the role of these agents in temperature regulation (173, 174, 175). Observations on their influence upon the oxygen consumption of liver slices have also been made (9, 59, 144). Work by Fishgold *et al.* (59) on rat cerebral cortex slices demonstrated that low concentrations of sodium salicylate increased whereas higher concentrations progressively decreased oxygen consumption. No significant effect on respiration was observed when acetylsalicylic acid was incubated with brain cortical slices. No stimulation of respiration could be obtained with rat liver slices by sodium salicylate under the same experimental conditions.

Recently, Sproull (209) has shown that graded concentrations of sodium salicylate (3.5×10^{-4} M to 2×10^{-3} M) increased the rate of oxygen consumption of mouse liver slices. At higher drug concentrations (3×10^{-3} M to 7.5×10^{-3} M) the mean Q_{O_2} of the treated tissues fell below that of the controls. The author placed emphasis on the similarity of the action of the salicylates and that of dinitrophenol.

2. Studies on mitochondria. The study by Sproull confirmed an observation (25) that the salicylates were uncoupling agents when tested in mitochondrial systems. Sodium salicylate and acetylsalicylic acid depressed the efficiency of oxidative phosphorylation of brain mitochondria by 45 and 25 per cent respectively. The P:O ratios of liver mitochondria are depressed by 65 and 50 per cent under the same conditions.

Liver mitochondria are apparently more sensitive to the uncoupling action of the salicylates than are brain mitochondria, whereas other uncoupling agents have a greater inhibitory effect on phosphorylation when incubated with brain mitochondria (30). Excess magnesium ion failed to reverse the inhibition produced by the salicylates. These antipyretics also inhibited fatty acid (octanoate) oxidation by rat liver mitochondria. Other related agents such as antipyrine, acetophenetidin and gentisic acid failed to depress the P:O ratio or inhibit fatty acid oxidation.

One can hardly attribute the antipyretic action of the salicylates to the uncou-

pling action on brain mitochondria on the basis of the present data. It is possible however that these effects on oxidative phosphorylation may account for the observed increase in total metabolic rate (40), the glycogenolytic action (210), the hyperglycemia (15), the increase in heat production (49) and the production of hyperthermia after brain stem transection (97). Thus, some but not all of the pharmacological actions of the salicylates may be explained by their ability to uncouple phosphorylation.

D. The Nitro- and Halophenols

The nitro- and halophenols have had a wide variety of uses. Originally, these compounds were thought to have some utility as substitutes for thyroid preparations since they increased metabolism, but because of their toxicity they were finally discarded. More recently the substituted phenols have had wide agricultural use as both insecticides and herbicides. Although their use as medicinal agents is no longer important, the toxicity of these agents to man and the mechanism by which they affect plants and insects is still the subject of considerable research. In the intact animal these agents cause an increase in oxygen consumption and a rise in body temperature. Death may result from respiratory failure or hyperthermia and the onset of rigor mortis is characteristically rapid. The pharmacology of these compounds has been reviewed by Edsall (53) and von Oettingen (169).

1. In vitro studies. a. Stimulation and depression of cellular respiration. It has been demonstrated repeatedly that low concentrations of 2,4-dinitrophenol (DNP) and related compounds augment cellular respiration when added to various types of tissue preparations (39, 54, 58, 123, 172, 179). Concentrations of DNP which stimulate respiration inhibit such processes as growth and cell division (112, 148). These latter effects were originally explained by Loomis and Lipmann (141) on the basis of inhibition of the formation of high-energy phosphate compounds. It is generally accepted that the observed stimulation is the result of the uncoupling of phosphorylation and oxidation.

Simon (204) has cautioned that the uncoupling property is not the only mechanism of toxic action attributable to the nitrophenols. If these agents are present in higher concentrations, oxidation and fermentation, as well as phosphorylation are inhibited. Unfortunately, these other actions of dinitrophenol have not been emphasized, with the result that any cellular process that is depressed by DNP is usually ascribed to the uncoupling action of this agent. The precise action of DNP is thus dependent upon the concentrations used, when one employs high amounts of this compound it is to be expected that several mechanisms of inhibitory action are operative.

In several studies (63, 113) the investigators failed to find the stimulation usually observed with DNP. In these experiments it is possible that the amount of agent which depresses respiration is only slightly higher or may even overlap the concentration which will produce stimulation. The increase in respiration may be masked by an inhibitory effect on the oxidative enzymes. This may explain the failure of certain uncoupling agents, such as the barbiturates, to stimulate oxygen consumption to the same degree as DNP (30).

b. Site of action. Just how DNP uncouples oxidative phosphorylation is still unknown and has been a point of conjecture for the past few years. The current concepts regarding the mechanism of DNP action have been reviewed by Simon (204) and Laties (128). In the presence of DNP the requirement of some oxidative enzymes for phosphate is abolished (141). This was interpreted to mean that DNP could replace inorganic phosphate in the same sense that arsenate could "replace" inorganic phosphate in aldotriosephosphate oxidation. Subsequent experiments, however, indicated that DNP did not act in the same manner as arsenate (218).

Some investigators have postulated that DNP exerts its effects by activating an ATP-ase. Although DNP does stimulate the anaerobic breakdown of ATP and pyrophosphate (238), this finding does not adequately explain its action. Dinitrophenol could also inhibit some transphosphorylation system, although studies thus far have demonstrated no such enzyme susceptible to DNP (238). It has been suggested by some workers (46, 93, 218) that DNP acts by causing decomposition of a primary, energy-rich, phosphorylated intermediate or enzyme during citric acid cycle oxidations. Since substrate phosphorylation appears to be insensitive to DNP (93), this uncoupling agent may only cleave the intermediate formed during respiratory-chain phosphorylation.

The last hypothesis seems to be the most tenable one at the present time, although the available evidence to support it is of an indirect nature. Further work in this field utilizing radio-tracer methods similar to those employed by Cohn (41) may aid us eventually to solve this problem of site of action.

2. In vivo studies. Investigations in the intact animal indicate that this class of compounds influences tissue nucleotide levels, tissue oxygen consumption and body temperature.

a. Tissue nucleotides. The evidence that tissue nucleotides are depleted by these agents is not convincing. Although there are data demonstrating that DNP depletes phosphocreatine in muscle (189) and brain (155) *in vitro*, these effects have not been unequivocally demonstrated *in vivo*. Stoner *et al.* (171) have pointed out that whereas a wide variety of agents can uncouple *in vitro*, many of these appear to be inert *in vivo*, or are slow to exert their full effect. The insecticide 3,5-dinitro-orthocresol (DNOC) uncouples oxidative phosphorylation *in vitro* and depletes brain adenosine polyphosphates and phosphocreatine in animals given fatal doses of the compound. Conversely, a chemically related compound, 2,4-dinitro-α-naphthol produces an increase in the phosphocreatine concentration in muscle (164). If DNP uncoupled *in vivo*, one might expect changes in the phosphate compounds of the plasma, but Tainter *et al.* (217) found the inorganic, organic and total phosphorus of the urine, blood and feces practically unchanged after the administration of dinitrophenol to man. These anomalous findings have not been reconciled.

b. Hyperthermia and increased tissue metabolism. The observation that DNP causes an increase in body temperature has been made by a large number of investigators (169) using many different species. The increased temperature is said to be closely associated with the increase in oxygen consumption observed with DNP. Hall *et al.* (79) showed a linear relationship between the rise in body

temperature and the increase in oxygen consumption. The stimulation of oxygen consumption usually preceded the hyperthermia indicating that the increased oxidation was responsible for the rise in temperature. Additional studies also indicate that the effect of DNP and related compounds on heat production is peripheral rather than central in origin. The hyperthermia is not influenced by anesthetics (145, 216), spinal transection or extirpation of the heat-regulating center in the hypothalamus (215).

However, a number of experiments with dinitro-α-naphthol suggest that the increase in body temperature is not solely the result of the increased metabolism. The hyperthermia with this compound was still observed after sub-bulbar transection (84) and could not be prevented by the administration of cyanide which effectively depressed the respiration (57, 222, 223). Thus there may be a central component to the hyperthermia which in the past has been attributed solely to the increased metabolism. It might be pointed out here that the pronounced hyperthermic action may be a property unique to uncoupling agents of the nitro- or halophenol series. The literature does not reveal that uncoupling agents unrelated to the phenols also evoke a hyperthermic response.

E. Thyroid Hormones

Since it is well-established that the thyroid hormone and related compounds increase the metabolism of tissues and animals, it would seem plausible that these agents might influence some oxidative or high-energy generating enzyme system. Although a considerable number of studies have been directed toward the elucidation of the mechanism of action of these agents, the precise site at which they act has not been demonstrated.

1. Comparison of the action of thyroid and the substituted phenols. The similarity in chemical structure of thyroxin and the halo- and nitrophenols and the observations that these agents increase body temperature encouraged comparative studies of these compounds. In general there are more differences than similarities.

Barker (16) showed that thiouracil-treated animals were less and thyroid-treated animals more responsive to the calorigenic action of the nitrophenols than were control animals and postulated that the substituted phenol might act via the thyroid hormone. While there appears to be some synergism between the two classes of compounds, the nitrophenols cannot replace thyroid hormone. This is indicated by the report of Dodds and Robertson (50) that DNOC did not relieve the symptoms of myxedema in man. DNP also fails to accelerate the metamorphosis of tadpoles (52) again indicating the differences in physiological response.

Several studies have shown differences in animals treated with these agents and subjected to various environmental temperatures. The hyperpyretic response can be demonstrated with DNP in animals at 27° C., while at 20° C. this compound produces no increase in body temperature. Nor is an increase observed at 6° C., instead DNP produces a fall in body temperature (66). One may contrast this DNP action with the effect of thyroxin on the body temperature of animals exposed to low environmental temperatures. Thyroxin significantly increases the

temperature of mice incubated at 5° C., while a hypothermia is obtained with DNP-treated animals under the same conditions (220).

While physiological concentrations of thyroxin or thyroid hormone have anabolic and growth-promoting effects in the intact animal, larger doses have a definite deleterious action. A hyperthyroid animal may exhibit such symptoms as weight loss, muscle weakness, increased metabolic rate and increased body temperature which are reminiscent of DNP-treated animals. It is possible, therefore, that at high drug concentrations, thyroxin and related compounds act like DNP and uncouple oxidative phosphorylation.

2. In vitro studies. The effect of thyroid hormones on *in vitro* systems has been recently reviewed by Barker (16) and Dutoit (52).

a. Tissue slices. Rohrer (188) found that oxygen uptake was greater than normal in kidney, liver and muscle isolated from animals treated with thyroid. Cerebral gray matter of hyperthyroid rats did not show this increase in respiratory rate which is exhibited by other tissues (33). Although many investigators have verified the finding that excised tissues from thyroid-treated animals show an increased oxygen consumption, few have conclusively demonstrated that thyroxin added to excised tissue increases the oxygen consumption of that tissue. Davis *et al.* (47) found that the addition of thyroxin to frog heart accelerates respiration. Other workers have reported that thyroglobulin increases the rate of oxygen consumption of tissues *in vitro* (36, 190, 234), but Williams-Ashman could not confirm these results (235). The addition of *l*-triiodothyronine, a compound which is more potent in accelerating tissue metabolism *in vivo* (78, 83, 219), does not stimulate the rate of oxygen consumption of liver slices or hemidiaphragms isolated from normal rats (237).

b. Mitochondrial preparations. Considerable research has been directed toward the demonstration of an action of thyroxin on the oxidative or phosphorylative enzymes in mitochondria. While some investigators have found that thyroxin does depress the P:O ratio, many have failed to find such an effect. In most instances, where an inhibition of phosphorylation has been demonstrated *in vitro*, the concentrations of thyroxin have been excessive.

Using liver mitochondria from normal and hyperthyroid rats, Lardy and Feldott (121) observed lower P:O ratios in mitochondria isolated from the hyperthyroid animals. These workers also found that thyroxin added *in vitro* depressed the P:O ratio of mitochondria that are oxidizing glutamate to succinate. The depression of phosphate esterification was detectable at thyroxin concentrations which had no significant effect on oxygen consumption. Profound effects on phosphate uptake were observed only when thyroxin, sufficient to depress the respiration, was used. No stimulation of respiration was observed. Triiodothyronine, (10^{-5} M), also uncoupled phosphorylation while having a minimal effect on oxygen consumption (146). Hoch and Lipmann (87) also demonstrated that phosphorylation is inhibited and oxidation increased in mitochondria obtained from thyroxin-treated rats as compared to untreated controls. Martius and Hess (149) showed that thyroxin, both *in vivo* and *in vitro*, inhibits the incorporation of P^{32} into ATP by rat liver mitochondria. Conversely, Judah and Williams-

Ashman (103) found no uncoupling action of either thyroxin or thyroglobulin *in vitro*.

The studies of Bain (13) have shed considerable light on the basis for some of the inconsistent results obtained when thyroxin is added to mitochondrial preparations. Using mouse, rat and rabbit liver mitochondria, he found that the addition of thyroxin *in vitro* resulted in a significant decrease in the ability of the system to esterify inorganic phosphate, and that this inhibitory action could be accentuated by preincubation of drug and enzyme preparation. The depression of the P:O ratio, like that produced by the tetracycline antibiotics, was dependent upon the concentration of magnesium ion in the reaction medium. The inhibition of oxidative phosphorylation could be reversed by magnesium concentrations of 24 micromoles/ml. or greater. Although *l*-thyroxin is considerably more potent than *d*-thyroxin *in vivo*, no differences in potency were observed in this system. Triiodothyronine was a somewhat more effective inhibitor of phosphorylation, while the effect of diiodothyronine was negligible. A thyroxin antagonist, 4-benzyloxy-3,5-diiodobenzoate, was a more potent uncoupling agent than any of the other compounds studied. Its inhibitory effects were not magnesium-reversible and its action resembled more closely that of dinitrophenol or the barbiturates.

From the above experiments we may conclude the following: 1) The mechanism by which thyroxin and related agents depress phosphorylation is different from that of dinitrophenol, more closely resembling the inhibitory action of the tetracycline antibiotics. 2) The failure to find a difference in the uncoupling potencies of the thyroxin isomers and of triiodothyronine may indicate that the uncoupling is not related to the normal, physiological responses observed with these agents. Ordinarily, growth and maturation are enhanced by thyroid and related compounds and since uncoupling implies a dissipation of useful energy, a correlation between these actions is not readily apparent. 3) It is conceivable, however, that the effects of large doses of thyroid or thyroxin seen in the intact animal (weight loss, hyperthermia, etc.) may be related to the inhibitory action of these agents on oxidative phosphorylation.

F. Miscellaneous Uncoupling Agents

A large number of compounds have been demonstrated to possess uncoupling action. The plant growth regulator, 2,4-D, will inhibit oxidative phosphorylation and stimulate the respiration of rat liver mitochondria in a phosphate-deficient system in concentrations of 10^{-4} to 10^{-3} M (24). Bonner could not show an uncoupling effect with this agent in plant tissues (23). Serotonin (12) and several parasympathomimetic drugs (4) have been shown to depress oxidative phosphorylation but in concentrations that far exceed those that would be pharmacologically relevant. At a concentration of 10^{-4} M, prostigmine inhibited phosphorylation by 50 per cent while diisopropylfluorophosphate inhibited about 80 per cent at 5×10^{-4} M (4). Although pentylenetetrazol injected in lethal doses depressed oxidative phosphorylation in brain homogenates by 20 per cent (4), no effect on the P:O ratio of brain mitochondria could be demonstrated using

drug concentrations up to 10^{-2} M (26). A cyanine dye specifically inhibits the renal tubular transport of N-methyl-nicotinamide and tetraethylammonium. This compound also uncouples oxidative phosphorylation but at concentrations greater than those affecting renal transport mechanisms (177). Arsenate (131), arsenite (131), atabrine (141), certain dyes (131, 141), azide (142) and the nitrites and nitrates (95) also apparently uncouple phosphorylation. No evidence exists, however, to indicate that this activity is related to the pharmacological effects produced by these agents *in vivo*.

G. Electrical Stimulation and Uncoupling

Recently a technique has been developed by means of which electrical impulses can be applied to tissue slices respiring in an oxygenated, buffered medium containing a suitable substrate (68, 157). McIlwain and his coworkers (114, 153, 156, 158, 159) found that such electrical stimulation of tissue slices leads to a significant increase in respiration and characteristic changes in phosphorus metabolism. This technique has been extended to include brain homogenates and mitochondrial preparations (3, 10). Abood *et al.* (3) have demonstrated that phosphorylation was depressed by electrical stimulation. Using P^{32}, it was found that phosphate uptake was inhibited 30 per cent by electrical pulses which doubled the oxygen uptake. This effect was presumed not to be the result of an increased break-down of esterified phosphate (1) although other workers have indicated that electrical stimulation will split the phosphate esters of brain slices (154). It has been postulated that the decreased phosphate esterification is effected via an inhibition of ATP formation (1). Others (166) have failed to confirm the finding that electrical stimulation depresses phosphorylation or stimulates oxygen consumption in cell-free systems. The above techniques have also been employed in studying drug action (60, 82), however, the pharmacological implications of these investigations are not yet apparent.

V. SUMMARY

Under normal circumstances the oxidative metabolism of living cells and of certain isolated systems leads to the synthesis of compounds containing high-energy phosphate bonds. These compounds are known to be essential for such functions as growth, muscle contraction, nerve conduction, etc. A number of substances are able to alter this relationship by depressing the formation of high-energy bonds without depressing simultaneously the oxygen consumption of the system. This dissociation of oxidation and phosphorylation is termed "uncoupling".

Many of the substances which have been shown to uncouple phosphorylation from oxidation are drugs which have been employed in experimental pharmacology and therapeutics. Whether the effectiveness of these substances as drugs is dependent upon their effectiveness as uncoupling agents is the consideration of this review. The fact that a substance has the ability to uncouple phosphorylation from oxidation does not indicate that it is devoid of other fundamental actions. The relative importance of uncoupling as a mechanism of action will differ, of

course, with different drugs. That uncoupling is of major importance in the action of certain drugs is at present a most attractive hypothesis.

With each succeeding step in our inquiry, our questions become more sophisticated and the answers to the questions become more specific. At first it was sufficient to say that certain drugs acted by "interfering with oxidation". Further study leads to the differentiation of substances which depress both oxygen consumption and phosphorylation from substances which depress phosphorylation but leave oxygen consumption relatively unaffected.

Study of these latter substances, the uncoupling agents, reveals further subgrouping: 1) those agents which stimulate oxygen consumption and are insensitive to magnesium ion changes (*e.g.*, dinitrophenol) and 2) agents which do not stimulate oxygen consumption while depressing phosphorylation and whose action is reversed by magnesium ions (*e.g.*, the tetracyclines). The significance of this difference remains to be explained.

Further study and formulation will surely reveal some of our present concepts to be naive. But so long as we consider the orderly flow of energy through cellular systems essential to normal function, we may consider important any agent capable of diverting this energy stream. It is from this frame of reference that the author has reviewed the phenomenon of uncoupling.

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