THE EFFECTS OF DRUGS ON ENZYME SYSTEMS

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I. INTRODUCTION

It would be presumptuous to attempt a complete review of the effects of drugs on enzymes and enzyme systems. All drugs have at least indirect effects on enzyme systems, and few enzymes are not affected ultimately by some drug. Let us therefore quickly state that the objective of this review is limited to an examination of a few instances of well-studied drug effects which have been attributed to specific drug-enzyme interactions. In addition, we have chosen to struggle a little with one of the oldest problems in the field, the mechanism of anesthesia. It is hoped that the examples selected will illustrate the variety of ways that enzymes may be affected by drugs and perhaps a few of the difficulties in trying to determine for certain whether a particular drug exerts its action through specific attack on a particular enzyme.

Of the earlier reviews of the action of drugs on enzymes particular mention may be made of that of Bernheim in 1946 (26a). In addition there are numerous other reviews on specific groups of drugs, many of which have appeared in this journal. Certain of these will be referred to in the body of this review. A recent

review by Röe (206a) is particularly relevant to the section on the treatment of methanol poisoning with ethanol, and Brody has reviewed uncoupling of phosphorylation by drugs (30c).

One of the best documented cases of drug-enzyme action, that of the anticholinesterases, has been omitted because of the numerous recent reviews and the fact that a full treatment would constitute an entire review in itself. The specific agents discussed include acetazoleamide (Diamox), disulfiram (Antabuse), ethanol in the treatment of methanol poisoning, anesthetic agents, penicillin, streptomycin, chloramphenicol, and the tetracyclines.

Before proceeding to individual cases, it may be appropriate to touch on some of the things that must be established before one is willing to conclude that any or all of the effects of a drug in the body arise from action on a specified enzyme. In general rigorous proof that a drug acts by inhibiting a particular enzyme would require demonstration 1) that the enzyme concerned is inhibited in the living intact tissue or cells, 2) that the enzyme block will quantitatively explain the effects of the drug, and 3) that the enzyme inhibition occurs with an amount of drug no greater than that necessary to produce the drug action under consideration. It is accepted that physostigmine, for example, exerts its action through inhibition of cholinesterases because (a) the natural substrate of cholinesterases accumulates in poisoned tissues, (b) the symptoms of poisoning may be approximated by acetylcholine if suitably applied, and (c), since the dissociation of the drug from the enzyme is very slow, poisoned tissues may be excised, ground up, and the enzyme inhibition confirmed in the test tube. If the drug-enzyme complex is rapidly dissociable, proof is more difficult. As a minimum it is then necessary to show (as has been shown with physostigmine) that the drug inhibits the enzyme concerned *in vitro* at concentrations consonant with the dosages effective *in vivo*. If a 10^{-8} molar drug concentration is required to inhibit a certain enzyme in the test tube, then obviously action on this enzyme will hardly explain effects in the animal produced with a dose of 10^{-5} moles per kgm. Enzymes as a class of proteins naturally have much in common, and it is not surprising if a potent drug with a selective action on one enzyme affects other enzymes when the concentration is increased a hundred fold.

If an isolated enzyme is inhibited by a drug at a concentration as low as that found in the tissues after therapeutic dosage, it still may not prove that the enzyme concerned is inhibited *in vivo*. It must first be established that the concentration of *free* drug in the tissue is sufficiently high to produce inhibition, since other cell constituents might bind a substantial fraction of the drug. For example, the oxidation of succinate in a liver homogenate is inhibited more than 90 per cent by 0.015 microgm./ml. of antimycin A if the tissue dilution is 1 to 300, whereas the same concentration of antimycin A inhibits oxidation by only 10 per cent in the presence of three times as much tissue (190).

It is conversely true that failure of a drug to inhibit an isolated enzyme may not prove that the enzyme activity concerned is unaffected *in vivo* by that drug, since enzymes are not always isolated without alteration in their properties and drug susceptibilities. It is also possible that a drug may be concentrated in the

body at the site of action and therefore produce a degree of inhibition greater than expected from *in vitro* measurements. However, without proof, this latter argument could only be a weak support for a sagging theory.

The above caveat is not intended to imply any lack of confidence in the ingenuity of the investigator to ultimately unravel these matters. Instead it might be taken as a tribute to the ingenuity and subtilty with which the enzyme machinery is contrived in the first place.

II. ACETAZOLEAMIDE

1. General. Diamox® (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide) has been introduced into the treatment of edema, glaucoma, and epilepsy. It appears to be one of the best examples of a drug acting through specific inhibition of a single enzyme. Although there seems to be little reason to doubt that most of the effects of Diamox result from inhibition of carbonic anhydrase, there remain interesting problems as to the exact function of this enzyme in, for example, the acidification of the urine.

The history of this drug and its antecedents has been an instructive sequence of clinical observations, physiological and biochemical studies, and deliberate attempts to synthesize more potent congeners. The results have been important for both therapy and basic physiology. Southworth (218) first recorded that sulfanilamide used as an antibacterial agent consistently lowered the CO_2 combining capacity of plasma. Basman and Perley (20) noted that with sulfanilamide "the pH of the urine quite regularly is above 7". Marshall *et al.* (154) confirmed these findings with dogs but also found that the plasma pH was slightly decreased and therefore ruled out hyperventilation as a possible cause of the alkaline urine and bicarbonate loss. Mann and Keilin (150) next discovered that sulfanilamide and a wide assortment of other sulfonamides are potent inhibitors of carbonic anhydrase, provided that the sulfonamide group is free.[1] The following year Davenport and Wilhelmi (148) found abundant carbonic anhydrase in kidney cortex, and in 1942 Höber (98) observed that sulfonamides cause prompt, reversible alkalinization of frog urine provided, again, that the sulfonamide group is free. Höber therefore concluded that the reabsorption of bicarbonate and the normal acidification of the urine "can be looked upon as being causally related to the catalyzing effect of carbonic anhydrase". Pitts and Alexander (188) found a drop in titratable acidity in the urine of dogs receiving sulfanilamide. Since they had good evidence that acidification of urine results from addition of hydrogen ions rather than from withdrawal of bicarbonate, they revised the interpretation of Höber, *i.e.*, carbonic anhydrase is necessary in order to accelerate the production of hydrogen ions from carbonic acid rather than to facilitate the reabsorption of bicarbonate.

Strauss and Southworth (221) had reported that patients receiving 5 gm. of

[1] Later studies of Krebs (128) showed that N^1 substituted sulfonamides, although much weaker than unsubstituted analogs, possess considerable anti-carbonic anhydrase activity. For example, the anhydrase is inhibited 50 per cent by 5×10^{-4} M sulfapyridine. This is five hundred times the concentration of free sulfanilamide required to produce the same result.

sulfanilamide per day experienced a diuresis and a doubling of sodium excretion with a less persistent increase in potassium excretion. The effects were not fully sustained longer than three or four days. Schwartz (211) used sulfanilamide with some success in the treatment of cardiac edema, and his results stimulated Roblin and Clapp (204) to try to prepare more potent and less toxic inhibitors of carbonic anhydrase. They succeeded in synthesizing several sulfonamides which were fifty to one hundred times as active as sulfanilamide (171). Berliner *et al.* (26) tested Diamox, one of the most potent and least toxic of these inhibitors, on dogs and found that, together with its enhanced potency as an anti-carbonic anhydrase, it is much more active than sulfanilamide in inhibiting renal acidification and in promoting excretion of Na^+ and K^+. Numerous subsequent studies have confirmed these findings and have extended them to patients under treatment. (See, for example, Maren *et al.* (152, and 153).)

Diamox is one of the most active enzyme inhibitors known. At 0°C. a concentration of the order of 10^{-8} M (2 microgm./l.) causes 50 per cent inhibition of carbonic anhydrase. Since five thousand times greater blood and tissue concentrations are easily attainable, it is inescapable that carbonic anhydrase may be profoundly depressed by Diamox *in vivo*.

Diamox is a reversible inhibitor (Maren *et al.*, 152), and it would be anticipated that a concentration of 10 microgm./ml., *i.e.*, five thousand times the Michaelis inhibitor constant, would suppress 99.98 per cent of the enzyme activity. Roughton (207), by extrapolation from measurements at lower temperatures, estimated that red cells contain sufficient enzyme to accelerate the hydration of CO_2 about one thousand five hundred fold at 38°. A 99.98 per cent inhibition would be expected to almost abolish red cell catalytic activity. Even the parietal cells, which may be richest of all cells in carbonic anhydrase (45) would be so inhibited that the residual enzyme activity would not more than triple the uncatalyzed rate.

Davenport (46) actually tested *in vitro* a concentrated solution of purified carbonic anhydrase which was about as active as red blood cells themselves. He added to this solution thiophene-2-sulfonamide, an anhydrase inhibitor which is about one-tenth as active as Diamox. He used an inhibitor concentration (2 mM) which was ten thousand times the inhibitor constant. The observed inhibition was 99.98 per cent as compared to a calculated inhibition of 99.99 per cent.

In spite of the apparent validity of such calculations, it has not yet been possible to determine with certainty the degree of depression *in vivo* of carbonic anhydrase by Diamox or related inhibitors. For a number of reasons, neither the direct measurement nor the indirect calculation of the degree of inhibition in intact tissues or blood has so far been satisfactory. Although one may measure the concentration of Diamox in the kidney, for example, it is likely that neither the enzyme nor the inhibitor is uniformly distributed among the various parts of the nephron, so that the concentration of Diamox in the anhydrase-containing cells is unknown. Even in the erythrocyte there has been great difficulty in assessment of enzyme inhibition. The uncatalyzed hydration of CO_2 is so rapid

at 38°C. that almost all measurements have been made at 15°C. or below—usually at 0°C. Some, at least, of the anhydrase inhibitors become less active at higher temperatures (128). Also, Krebs found that the presence of heat-inactivated renal cortex increased the quantities of various sulfonamides which were required to produce a given degree of inhibition. Thus extrapolation to living tissues at 38°C. from measurements with a dilute enzyme system at 0°C. may be misleading.

Miss Elizabeth Crawford in this laboratory was kind enough to conduct a few orientation experiments with carbonic anhydrase of lysed human red blood cells and with Diamox at 38°C. (44a). The measurements were made in 0.16 M KCl. With phenol red as an indicator the time was measured for the solution to change from pH 7.8 to 7.1 with an initial CO_2 concentration of only about 2 mM, *i.e.*, an attempt was made to make measurements under conditions not too remote from those existing in the red cell. The results indicated that red cells may actually accelerate the hydration of CO_2 eight thousand fold and that the inhibitor constant for Diamox under these conditions is about 6×10^{-8} M. If these data are correct, a plasma level of 10 microgm. Diamox per ml. (5×10^{-5} M) would still permit the red cells to accelerate the hydration of CO_2 ten fold.

The red cell, kidney, stomach, pancreas, and ciliary body have the following in common: 1) they must transport large amounts of acid or base out of the blood plasma (the acid being CO_2 in the case of the red cell), 2) they are unusually rich in carbonic anhydrase (47, 247), and 3) their acid base functions are inhibited by Diamox. Each of these tissues will be discussed in regard to the effects of Diamox. In addition, the action of Diamox on brain will be noted, although the significance of carbonic anhydrase for cerebral function is not clear.

2. Red blood cells. Some experiments of Davenport (46) with intact red blood cells have been taken by others to indicate that the sulfonamide inhibitors are much less active inside of cells than in homogeneous solution. Davenport measured CO_2 uptake by 3 ml. of whole blood at 0°C. after adding various amounts of sulfanilamide or thiophene-2-sulfonamide. With concentrations of the thiophene derivative greater than 25 mgm. per cent, CO_2 uptake was slow and constant and this was assumed to represent the uncatalyzed rate of CO_2 hydration. Without inhibitor the rate was seventeen times faster. It required 10 mgm. per cent inhibitor to reduce CO_2 uptake to three times the uncatalyzed rate. This he regarded as only an 87 per cent inhibition, whereas a 99.97 per cent inhibition would be expected from the inhibitor constant. Similar experiments with sulfanilamide at various levels were in keeping with the example cited.

It is possible to reinterpret Davenport's experiment to indicate that the enzyme in the intact red cell was in fact inhibited to the extent predicted from the inhibitor constant (Table I). It will be noted that although the uninhibited red cells only accelerated CO_2 uptake seventeen fold, a ten thousand fold acceleration was to be expected at 0°C. (207). It seems possible that in the large scale experiment performed, factors other than carbonic anhydrase were rate limiting until a very large fraction of the enzyme was inhibited. In this case, the three fold acceleration of CO_2 uptake which persisted in the face of 10 mgm. per cent thio-

TABLE I

*Carbonic anhydrase inhibitors on the CO_2 uptake by intact red blood cells**

Thiophene-2-sulfonic acid			Sulfanilamide		
Inhibitor concentration	Acceleration of CO_2 uptake		Inhibitor concentration	Acceleration of CO_2 uptake	
	Observed	Calculated		Observed	Calculated
mgm. per cent			*mgm. per cent*		
0	17	10,000	10	8	17
1	6	26	25	6	7
10	3	3	50	3.5	3
25	0	1	125	2	2
			250	1	1

* Davenport (46).

phene-2-sulfonamide might represent three out of a possible ten thousand or an inhibition of 99.97 per cent, as predicted, instead of three out of a possible seventeen. The other data in the table are in accord with this interpretation.

Diamox enters red cells promptly after administration and attains a concentration which approximates that of plasma. After the plasma level has fallen to less than 1 microgm./ml. the drug still persists in red cells at concentrations of 5 to 30 microgm./ml. for twenty-four hours (152). One possibility for this persistence is that there is enough carbonic anhydrase in the red cell to retain this amount of Diamox as long as the plasma level is sufficient nearly to saturate the enzyme. It has been estimated that the enzyme accounts for about 0.7 per cent of red cell solids (116) or about 2.5 mgm./ml. of red cells. The molecular weights of the enzyme and drug are respectively 30,000 and 222. Therefore, with one molecule of drug per molecule of enzyme, red cells could retain nearly 20 microgm. of drug per ml. in enzyme combination with as little as 0.1 microgm. Diamox per ml. of plasma. Maren *et al.* (152) failed to obtain the expected inhibition of very high concentrations of carbonic anhydrase by Diamox. It seems possible that there may have been present in the test system more enzyme than inhibitor on a molar basis, in which case only partial inhibition could occur.

In considering the effects of Diamox on the red blood cells, the problem is not to explain the mechanism of action, but to demonstrate whether or not there *is* an action, *i.e.*, whether CO_2 transport is inhibited by the drug. Roughton calculated that the uncatalyzed rate of dehydration would need to be accelerated one hundred fifty fold to explain the known rate of CO_2 unloading in the lungs. Yet animals survive with concentrations of Diamox in their red cells which would be expected to inhibit carbonic anhydrase 99.99 per cent. It is true that at these levels there is evidence of increased difficulty in removal of CO_2 through the lungs. The plasma CO_2 tension may increase in spite of the low bicarbonate concentration (153) and increases consistently if the renal effects of Diamox are prevented with NH_4Cl (151). Tomashefski *et al.* (227) found that with 25 to 100 mgm. Diamox per kgm. injected intravenously into dogs, there occurred within thirty minutes a 25 per cent fall in alveolar pCO_2 and a 20 per cent rise in arterial pCO_2. The authors suggest that the rise in pCO_2 measured in the drawn blood

sample may be a kind of artifact. That is, after drawing the blood extra CO_2 may have been formed from carbonic acid and bicarbonate which had not been dehydrated as rapidly as usual in the pulmonary capillaries.

In spite of this positive evidence of interference with red cell function by Diamox, Tomashefski *et al.* found that *oral* doses of 25 mgm./kgm. produced no demonstrable interference with CO_2 elimination in man or dog. This confirmed the previous careful study of Becker *et al.* (23) who gave 50 mgm. Diamox per kgm. to human beings without changing arterial or alveolar pCO_2, even after exercise. Presumably intravenous injection of Diamox produces higher maximum concentrations in the red cells than the same doses given orally, although Maren *et al.* (152) found the route made little difference in red cell or plasma levels after an hour. The only report of interference in gas exchange in man is that of Shepard *et al.* (212) who found a moderate increase in arterial-alveolar pCO_2 difference with Diamox and heavy exercise.

The meager effects of moderate doses of Diamox on CO_2 elimination are in keeping with the unpublished results cited above that the activity of human carbonic anhydrase and the inhibitor constant for Diamox are both greater at 38°C. than previously supposed, and that short of extreme Diamox dosages enough carbonic anhydrase is present to permit normal unloading of CO_2 in the lungs. Even with complete suppression of anhydrase, there are possibilities for adjustment to permit adequate CO_2 elimination. Thus, it may be that an increased fraction of CO_2 is transported as carbamino CO_2 and as free unhydrated CO_2. Also, because of hyperventilation, a greater proportion of this CO_2 could be unloaded at the lungs. In addition, the inhibition of carbonic anhydrase should cut both ways. That is, it should delay hydration of CO_2 added to the blood by the tissues as well as delay dehydration of bicarbonate in the lungs. Therefore, with extreme anhydrase depression, some of the CO_2 from the tissues might persist as such until the blood reaches the lungs. This would mean that the pCO_2 under these circumstances would be momentarily higher in the pulmonary capillaries than in a drawn venous blood sample analyzed at leisure.

3. Kidney. For the effects of Diamox on renal function the reader is referred especially to the original paper of Berliner *et al.* (26) as well as that of Maren *et al.* (153). It is the purpose here merely to explore the reasonableness of the view that inhibition of carbonic anhydrase is the mechanism by which Diamox and related substances inhibit renal acidification. It has been pointed out that among the sulfonamides tested only those which inhibit carbonic anhydrase affect renal function. The relative effectiveness of action against carbonic anhydrase and renal acidification are found to be parallel, at least with the three drugs most thoroughly tested, sulfanilamide, thiophene-2-sulfonamide (47), and Diamox. Pitts and Alexander (188) found that the titratable acidity of dog urine was reduced to half by sulfanilamide sufficient to produce a blood level of about 300 microgm./ml. With Diamox, 50 per cent of the maximal effect is produced at blood levels of the order of 2 microgm./ml. (153). The ratio of these plasma levels is not far from the ratio of the respective inhibitor constants for carbonic anhydrase.

Maren *et al.* (152) measured the concentration of Diamox in the kidneys of rats receiving 100 mgm. Diamox per kgm. per day. A concentration of 10 microgm./gm. was found, which if the drug were evenly distributed, would be expected to depress renal carbonic anhydrase by more than 99.9 per cent.

Since the Diamox inhibition of anhydrase is rapidly reversible, it is clear that the degree of inhibition in homogenates of tissue from an animal which has received the drug will vary with the dilution. The extent of inhibition observed in a dilute homogenate will be far less than the inhibition *in situ*. A few investigators working with renal tissue have overlooked this fact with resultant grossly low estimates of the amount of carbonic anhydrase inhibition in the intact organ.

A single dose of Diamox will completely suppress renal acidification, and there results a large loss of HCO_3^-, Na^+ and, usually, K^+ in the urine. Nevertheless, if drug administration is continued, the kidney begins again to acidify the urine and conserve fixed base. This is coincident with a substantial fall in plasma pH and HCO_3^- concentration (153). Experimental animals may be maintained thereafter for weeks or months with relatively high doses of Diamox without progressive loss of fixed base. However, as long as Diamox is given, the pH and HCO_3^- of the plasma remain low. The effect of Diamox in blocking acid excretion may also be overcome by inducing a severe acidosis with NH_4Cl (151). When 12 mEq. NH_4Cl per kgm. were given, the output of acid plus NH_4^+ was five times normal and was not affected by Diamox (153).

These results seem to show that carbonic anhydrase is not an obligatory component of the renal acidification mechanism. Perhaps instead it permits the kidney to produce acid under normal conditions of pH and CO_2 tension. Whatever the mechanism of renal acidification, carbonic acid must be the ultimate source of hydrogen ions needed (188), and the hydration of CO_2 is required before these hydrogen ions are available. With the development of metabolic acidosis by any means, the CO_2 tension in the cells of the kidney might increase to the point where the uncatalyzed hydration rate would suffice for acidification purposes.

4. Stomach. Earlier, less potent anhydrase inhibitors failed to inhibit gastric hydrochloric acid production in the mammal (57), although Davies and Edelman (49) blocked acid formation in isolated frog stomach with 10^{-2} M thiophene-2-sulfonamide and 10^{-3} M Prontosil Soluble.[2] Janowitz *et al.* (108) administered Diamox to three dogs with Heidenhain pouches and measured the gastric secretion resulting from histamine stimulation. They found that 20 to 120 mgm. Diamox per kgm. (i.v.) depressed the volume of juice about 60 per cent and the acid production about 75 per cent. For some collection periods the depression was over 90 per cent but was never complete. Furthermore, there seemed to be no greater effect from the drug at 120 mgm. than at 20 mgm./kgm., suggesting that some acid production might persist with complete suppression of carbonic anhydrase. Lower dosage (5 to 10 mgm./kgm.) depressed gastric secre-

[2] Prontosil, one of the earliest sulfonamide drugs, is one of the most potent anhydrase inhibitors, rivaling Diamox; however, it is converted to sulfanilamide *in vivo* and therefore loses most of its activity.

TABLE II

*Effect of Diamox on pancreatic secretion in the dog**

Values were obtained 3 hours after Diamox administration, except with 30 mgm./kgm. level which is a 2-hour measurement. Flow was stimulated with secretin.

Dose of Diamox	Volume output	Bicarbonate output	Bicarbonate concentration
mgm. per kgm.	*per cent of control*	*per cent of control*	*mEq. per l.*
2	76	75	125
5	87	76	107
10	40	27	69
30	61	46	95
40	21	14	78
60	30	22	91

* Birnbaum and Hollander (29).

tion in only one of three dogs. It is clearly more difficult to affect gastric secretion than renal acidification by carbonic anhydrase inhibition.

5. Pancreas. As with gastric secretion, pancreatic secretion is not inhibited by large doses of sulfanilamide (230). Nevertheless, Birnbaum and Hollander (29) found that Diamox in sufficient quantity can depress both volume and bicarbonate output of the pancreas (Table II). It will be noted that suppression was not complete and that 10 mgm. Diamox per kgm. was about as effective as 60 mgm./kgm. A dose of 10 mgm./kgm. would give a plasma level of 8 or 10 microgm./ml. at three hours (152). This would be expected to inhibit carbonic anhydrase as much as 99.9 per cent as judged by *in vitro* tests. It is possible that the uncatalyzed rate of carbonic acid hydration is sufficient to permit 20 or 30 per cent of the normal pancreatic secretion of HCO_3^-.

6. Ciliary body. The favorable effects of Diamox on glaucoma (22) seem to represent still another case of a carbonic anhydrase inhibitor acting on a structure (ciliary body) which does acid-base work. The concentration of bicarbonate in the aqueous humor of the posterior chamber is at least 40 per cent greater than that of plasma (121). Dr. Bernard Becker (22a) gave Diamox to a series of fifteen rabbits which had been nephrectomized to eliminate possible renal effects. Six hours later the bicarbonate in the posterior chamber fluid had fallen from 167 per cent of the plasma level to 135 per cent of the plasma level (the latter remained unchanged). Simultaneously the rate of aqueous flow decreased 64 per cent. Green *et al.* (77) have shown that Diamox given intravenously will inhibit carbonic anhydrase of the anterior uvea more than 90 per cent. It seems evident that for some unknown reason the rate of aqueous secretion is dependent on the ability of the ciliary body to concentrate bicarbonate, which in turn appears to depend in part at least on carbonic anhydrase. Increasing the Diamox dosage above 25 mgm./kgm. (rabbits) does little further to suppress the flow of aqueous humor or its bicarbonate concentration (Becker 22a). This suggests, as in the case of the stomach and pancreas, that the uncatalyzed CO_2 hydration rate is sufficient to permit a substantial fraction of the normal function.

7. Brain. Diamox is capable of decreasing the incidence of epileptic seizures

(25). Although this might be attributed to the favorable effects of acidosis in epilepsy, Diamox will raise the electroshock seizure threshold in experimental animals before there has been time for substantial bicarbonate loss (172). Theoretically at least, an increase of pCO_2 might occur rather promptly due to the effect of Diamox on the red cell. Therefore, it is not possible to say for certain that the increase in threshold is due to inhibition of brain carbonic anhydrase. Nevertheless, the authors made an interesting observation that suggests a direct action of the inhibitor on the brain. Sulfanilamide and Diamox are both effective in raising the electroshock seizure threshold. They found that Diamox is only twice as effective as sulfanilamide instead of being a hundred times more potent as anticipated from effects on the kidney and isolated carbonic anhydrase. Because of this discrepancy, Millichap and Woodbury (172) therefore measured the tissue concentrations of sulfanilamide and Diamox and found that sulfanilamide was fifty times more concentrated than Diamox in the brain at effective dosages. In spite of this suggestive evidence, there is still the possibility that Diamox and sulfanilamide act on the brain by a mechanism other than inhibition of carbonic anhydrase.

An observation which may be pertinent to the effects of Diamox on the brain has been made by Tschirgi *et al.* (229). They found that Diamox inhibits the rate of flow of spinal fluid even though this fluid normally does not differ very much from blood plasma in bicarbonate concentration or pH.

In summary, there is good evidence that in kidney, stomach, pancreas, and ciliary body Diamox interferes with the capacity to excrete or secrete acid or base through inhibition of carbonic anhydrase. Uncertainties about Diamox action on these structures center around the relationship of carbonic anhydrase to acid-base work and may not be removed until the mechanisms of acid and base secretion are understood. Present evidence suggests that although carbonic anhydrase facilitates the transport of acid or base it is not essential for this process. The evidence that Diamox affects the electroshock seizure threshold through inhibition of carbonic anhydrase is less satisfactory. In the case of red blood cells, the problem is to explain why gas exchange remains little affected by Diamox levels which would be predicted to profoundly depress the red cell carbonic anhydrase activity.

III. DISULFIRAM

Within a short time after the introduction of disulfiram (Antabuse®) for the treatment of alcoholism (85) its mode of action seemed established. *In vitro* tests showed that minute amounts of disulfiram can inhibit liver aldehyde oxidase (122). This appeared to explain the rise in blood acetaldehyde after ethanol and disulfiram (84), and the rise would in turn explain the symptoms, since acetaldehyde injection induces at least some of the unpleasant effects (9).

Although it is possible that this simple picture may prove to be nearly correct, subsequent studies have demonstrated a rather complex situation and many apparent discrepancies between theory and fact which must be cleared up before the disulfiram-ethanol reaction can be fully understood. Possibly some of the troubles arise from analytical difficulties in the measurement of acetaldehyde.

One of the first points to be investigated was whether disulfiram accelerates the oxidation of ethanol to acetaldehyde or slows the removal of acetaldehyde or both. Hald *et al.* (86) found that large doses of disulfiram actually inhibited alcohol oxidation, whereas with 0.75 gm. disulfiram or less per rabbit there was no effect on the rate of ethanol disappearance from the blood. (An increase in blood acetaldehyde was observed with as little as 0.25 gm. disulfiram per animal.) Thus the rise in acetaldehyde after alcohol and moderate doses of disulfiram seemed to be due to an inhibition of acetaldehyde oxidation.[3]

Nevertheless the effects of disulfiram on the removal of injected acetaldehyde from the blood are not as striking as might be anticipated. With rapid injection rates the diminution was so slight as to escape detection (9). Later it was found that when rabbits were infused with acetaldehyde at 2, 4, and 8 mgm. per minute, for example, the blood levels of acetaldehyde were respectively 0.1, 1, and 3.5 mgm. per cent in normal rabbits and 1, 2, and 5 mgm. per cent in rabbits that had received disulfiram (89). It thus appears that rabbits treated with disulfiram can metabolize almost as much acetaldehyde as before treatment, but that higher blood levels—and presumably tissue levels—are required. These findings were confirmed by liver perfusion experiments (87). With 1 or 2 mgm. per cent acetaldehyde in the perfusing blood, the control liver utilized three or four times as much acetaldehyde as the poisoned liver, whereas with 10 mgm. per cent acetaldehyde the rate of utilization was about the same for both. A somewhat comparable result was obtained by perfusion of the hind limbs, showing that neither the acetaldehyde rise nor the disulfiram effect is entirely limited to the liver. There was an upper limit to the rate of uptake by perfused liver, but an upper limit was not demonstrated in the experiments on the whole animal. Further confirmation of these results is to be seen in the fact that disulfiram does not materially change the LD_{50} for acetaldehyde (43, 181).

Disulfiram has two obvious properties that might suit it to the inhibition of certain enzymes. It forms a tight complex with copper as does its reduction product, diethyldithiocarbamate. (In fact, the latter is used to tie up copper in reactions where this metal is undesirable.) Secondly, disulfiram rapidly oxidizes reduced glutathione (110), and therefore might oxidize sulfhydryl groups of enzymes. The fact that diethyldithiocarbamate has the same *in vivo* activity as disulfiram (88) does not rule out this possibility, since the reduced compound might very well be oxidized to disulfiram in the body.

There are at least six enzymes in the mammal which are able to oxidize or otherwise dispose of acetaldehyde (Table III). Two of these are flavoproteins, two are DPN enzymes, and one contains thiamine pyrophosphate. The first four enzymes listed have been tested with disulfiram. Kjeldgaard (122) found

[3] There is an interesting paradox that has emerged from the studies of acetaldehyde formation from ethanol. The Danish investigators have found with intact animals (88, 134) and with perfused livers (107) that acetaldehyde formation is proportional to the ethanol concentration in the blood. This is very difficult to understand if the rate of ethanol oxidation is independent of concentration, a fact which these workers themselves have confirmed. This correlation is especially marked in the presence of disulfiram, but is found even in control animals receiving ethanol only.

TABLE III

Mammalian enzymes which attack acetaldehyde

	Location of greatest total activity	Coenzyme or prosthetic group	Product	Inhibitor constant*
Aldehyde oxidase	Liver	Flavinadeninedinucleotide	Acetate	2×10^{-7} M
Xanthine oxidase	Liver, intestine	Flavinadeninedinucleotide and molybdenum	Acetate	10^{-3} M
Aldehyde dehydrogenase‡	Liver	DPN	Acetate	10^{-6} M
Glyceraldehyde-3-phosphate dehydrogenase	All tissues	DPN, glutathione	Acetyl phosphate	5×10^{-6} M
Carboligase§	Muscle	Thiamine pyrophosphate	Acetoin, CO_2	†
Aldolase	All tissues	None	Pentose phosphate	†

* These values, which indicate the concentration of inhibitor required to produce 50 per cent inhibition, are only approximate and tentative since the inhibitions may be irreversible.

† No studies discovered.

‡ Racker (199).

§ Stotz *et al.* (220).

that 2×10^{-7} M disulfiram caused a 50 per cent inhibition of partially purified liver aldehyde dehydrogenase. Since an increase in substrate resulted in less inhibition, the author states that the inhibition "might be competitive in nature". However, this may not be the case. Kjeldgaard found that reduced disulfiram (diethyldithiocarbamate) is non-inhibitory and that reduced glutathione would not reactivate the disulfiram-inhibited enzyme. Since glutathione rapidly reduces disulfiram to diethyldithiocarbamate, the disulfiram inhibition is probably irreversible and therefore could not be competitive in the usual sense. Perhaps, instead, the substrate is able to protect the enzyme from the inhibitor.

Disulfiram is also able to inhibit liver xanthine oxidase (201) but it requires a high concentration of drug to produce a 50 per cent inhibition (Table III). A rather interesting finding was that no inhibition occurred if the electron acceptor was methylene blue instead of oxygen. Furthermore, after heating the enzyme to 56°C. for a few minutes, it was no longer inhibited by disulfiram even with oxygen as electron acceptor. That is, the disulfiram may not inhibit the first step of aldehyde oxidation by the enzyme, but may interfere with the subsequent reoxidation of the enzyme by oxygen. This step may be a more complex reaction than reoxidation by a dye. The effect of heating is not clear. Although xanthine oxidase acts as a unit, it is probably a small particle which constitutes a simple enzyme "system" rather than a single catalytic activity. Heating may disorganize the system and permit a short-circuiting of the disulfiram-sensitive step. Although these studies are interesting, they tend to rule out xanthine oxidase as an im-

portant point of action of disulfiram *in vivo* because of the comparative insensitivity to the drug. Nowinski and Ewing (177) also found that pigeons, which have no xanthine oxidase, nevertheless respond to disulfiram and ethanol with a rise in blood acetaldehyde as do many animals.

Graham (75) found that liver aldehyde dehydrogenase is strongly inhibited by disulfiram. His data indicate inhibition which is independent of the substrate concentration but which may be competitive with DPN. The data are compatible with reversibility, but the inhibition failed to pass the test of reversibility recommended by Ackermann and Potter (5). That is, the degree of inhibition was not independent of the amount of enzyme present but varied inversely with the concentration of the enzyme. This suggests that the apparent competition between disulfiram and DPN may in reality reflect a protective action by DPN. It is of interest that glutathione was able to "restore" the enzyme activity, although it is not clear from the protocols whether protection, competition or restoration is involved.

Kjeldgaard (122) stated that triosephosphate dehydrogenase is not inhibited by disulfiram. Nevertheless, Nygaard and Sumner (178) in an extensive paper demonstrated that muscle glyceraldehyde-3-phosphate dehydrogenase is quite sensitive to disulfiram ($K_i = 5 \times 10^{-6}$ M). Although this enzyme oxidizes acetaldehyde much more slowly than glyceraldehyde phosphate (93), it is a very abundant enzyme in muscle and the investigators suggest that it may be significant in the oxidation of acetaldehyde in the body. Nygaard and Sumner found that the inhibition data are compatible with competition with substrate, but not with DPN, *i.e.*, just the opposite of the results of Graham with liver aldehyde dehydrogenase. Again, the inhibition does not seem to be truly reversible, since when the enzyme concentration was varied the degree of inhibition also varied (inversely).

Krimsky and Racker (129) found that glutathione is the prosthetic group of triosephosphate dehydrogenase and presented convincing evidence that aldehydes combine with the thiol group of glutathione in a thioacetal link prior to oxidation by DPN (200). In view of the finding of Johnston (110) that disulfiram rapidly oxidizes reduced glutathione, the mechanism of disulfiram inactivation becomes rather obvious. It is tempting to suggest that acetaldehyde also combines with aldehyde dehydrogenase and aldehyde oxidase at an SH group. This would explain why these two enzymes are also susceptible to disulfiram inhibition.

In addition to the six enzymes listed in Table III, it may be added that alcohol dehydrogenase itself is reversible and that the equilibrium greatly favors conversion of acetaldehyde to ethanol. Finally, Kendal and Ramanathan (117) have raised the possibility that ethanol and acetaldehyde may form a semiacetal susceptible to oxidation to ethyl acetate by alcohol dehydrogenase.

With so many enzymes capable of removing acetaldehyde, it is not easy to establish the relative importance of each. Lubin and Westerfeld (142), by injecting acetaldehyde into cats after removal of various organs, were able to establish that the liver is the most important single site of acetaldehyde metabo-

lism. Their data suggest that of the total acetaldehyde which is removed from the blood stream, the liver accounts for 60 to 70 per cent, the other viscera about 10 per cent and the rest of the carcass 20 to 30 per cent. The data of Hald *et al.* (87) obtained by separate perfusion of rabbit liver and hind limbs are in accord with these results. When the liver is removed (142) substantial quantities of acetoin are formed, indicating that carboligase ("animal carboxylase") could be a significant pathway for acetaldehyde metabolism if the liver enzymes are blocked.

It is to be hoped that from a knowledge of the *in vitro* sensitivity of these enzymes one can decide whether the amounts of disulfiram used therapeutically or experimentally are sufficient to produce inhibition of any or all of these enzymes *in vivo*. In a rat given disulfiram tagged with S^{35} (55) and examined twenty-four hours later, S^{35} concentration was of the same order of magnitude in blood and in tissues. It was not established how much of the S^{35} represented unchanged disulfiram. Eldjarn (56) found a serum level of 3×10^{-5} moles of disulfiram per liter and 1.5×10^{-5} M diethyldithiocarbamate six hours after a patient received a 2 gm. dose of disulfiram (10^{-4} moles per kgm.). Divatia *et al.* (50) observed blood levels averaging 1.3×10^{-5} moles per liter and ranging from 0 to 9×10^{-5} M in patients receiving 0.25 to 1 gm. disulfiram per day. Such levels would be consistent with marked inhibition of three of the aldehyde oxidizing enzymes discussed above. However, the protective action of reduced glutathione and presumably of sulfhydryl groups in general might require the presence of higher levels for inhibition, whereas the possibility that the inhibition is irreversible might mean that lower concentrations would suffice.

If the disulfiram inhibition is irreversible or slowly reversible, it would be legitimate to prepare homogenates from poisoned animals and test the various enzyme activities *in vitro*. The only activity tested this way, to our knowledge, is xanthine oxidase, and equivocal results were obtained (202).

In spite of the enzyme complexities and the uncertainties of the exact conditions in the intact tissues, it is reasonable to assume that disulfiram inhibits acetaldehyde oxidation in both liver and muscle by inhibiting one or another of the enzymes concerned. There arises unfortunately one last obstacle to the acceptance of the original theory of the reaction to disulfiram plus alcohol. Some investigators believe that the blood acetaldehyde levels do not appear to be high enough to explain the symptoms, and that there is not very good correlation between symptoms and acetaldehyde concentration in the blood.

MacLeod (146) injected acetaldehyde into rats and found that to produce severe intoxication a blood level of 10 to 15 mgm. per cent acetaldehyde was required. When blood levels of 4 to 6 mgm. per cent were induced, there were few symptoms. In contrast, rats which were given disulfiram and ethanol sufficient to produce marked and prolonged symptoms had blood levels of only 0.8 to 1.9 mgm. per cent. There may be, of course, a difference in response in experimental animals and men.

As described in one of the original papers, Asmussen *et al.* (9) injected acetaldehyde into volunteers and obtained an increase in pulse rate, flushing and hyper-

ventilation with low acetaldehyde blood levels (0.55 and 0.7 mgm. per cent). They found, as had earlier investigators, that acetaldehyde produces a rise in blood pressure, whereas with disulfiram and ethanol the blood pressure falls, especially the diastolic pressure. Christensen (44) observed that a single injection of acetaldehyde in dogs gives a biphasic pressure response resembling very closely that produced by epinephrine. He found that disulfiram exaggerated the hypotensive phase for both epinephrine and acetaldehyde, and he therefore suggests that this may explain the fall in blood pressure in the disulfiram-ethanol reaction.

Hine *et al.* (97) noted a poor correlation between severity of symptoms and acetaldehyde levels as well as considerable overlap between the acetaldehyde levels observed after alcohol alone and those found in the group which had received both disulfiram and ethanol. Yet only those in the latter group showed the typical symptoms. Raby (198) in an extensive series found an average blood acetaldehyde level of 1.04 mgm. per cent (range 0.59 to 1.7) after alcohol alone (60 ml. average dose), and an average of 1.35 mgm. per cent (range 0.64 to 2.51) after disulfiram and ethanol (32 ml. average dose). Only those receiving both drugs showed symptoms other than those expected from the ethanol. The severity of the symptoms in the disulfiram group were correlated only very roughly with the aldehyde blood levels.

If the symptoms with disulfiram and ethanol are due solely to acetaldehyde, it is difficult to understand why the persons receiving alcohol alone did not also show symptoms. Raby noted that, in spite of the overlap in acetaldehyde levels, only in the case of those receiving disulfiram was there an odor of acetaldehyde on the breath. This certainly suggests that the method used by virtually all workers for the determination of acetaldehyde (219) may not be sufficiently specific. When used in disulfiram studies the Stotz method has not always been preceded by a distillation step as originally recommended. Furthermore, it is possible to form as much as 0.5 mgm. per cent acetaldehyde from ethanol present during deproteinization of whole blood with tungstate (146). Hald *et al.* (86) found that the actual output of acetaldehyde in alveolar air by rabbits receiving ethanol was increased two hundred to four hundred and forty fold by disulfiram, and yet the apparent blood levels differed only fifteen to twenty-five fold.

Thus disulfiram may cause a greater percentage rise in blood acetaldehyde than indicated by blood analyses. Possibly something appears in the blood during ethanol metabolism which is not acetaldehyde but which reacts like it in the Stotz procedure. A semiacetal, for example, between aldehyde and alcohol would break down during the distillation process to yield acetaldehyde. Kendal and Ramanathan (117) have given evidence of such a compound being formed during the oxidation of aldehydes by the liver.

It is necessary to end the disulfiram discussion on this somewhat uncertain note. It is definitely possible that disulfiram inhibits not only aldehyde oxidizing enzymes but other enzymes as well, and that in the presence of both ethanol and acetaldehyde these other enzyme deficits become manifest. Reichert *et al.* (202) found that disulfiram is capable of irreversible inhibition of the succinoxi-

dase system, which would clearly have far-reaching results. Likewise, inhibition of triose phosphate dehydrogenase would interfere with glycolysis as well as aldehyde oxidation.

IV. ETHANOL IN THE TREATMENT OF METHANOL POISONING

There seems to be general agreement that the toxicity of methanol is not due to the unchanged molecule but to its metabolites, formaldehyde and formic acid, especially the latter. As a rule there is a delay of twelve to forty-eight hours before the onset of serious symptoms, at which time a profound acidosis is regularly present. From 65 gm. of methanol, a dose likely to be fatal, could come 2000 millimoles of formic acid over a two or three day period. This flood of acid would be expected to overwhelm the acid-base defenses. However formate is partially oxidized in the body, and the formate actually excreted seems far too little to cause serious base depletion (53, 135, 143, 189). Instead the loss of fixed base appears to result from excretion of other acids, largely undetermined. For example, a patient studied by Van Slyke and Palmer (242) and by Harrop and Benedict (92) excreted per liter of urine 204 mEq. of organic acid consisting of 27 mEq. formic acid, 48 mEq. of ketone bodies, 17 mEq. of lactic acid, and 123 mEq. of acids of undetermined nature. The plasma bicarbonate was only depressed to about 60 per cent of normal and the patient recovered.

If methanol *per se* is not very toxic, it is reasonable that anything which might inhibit its oxidation would be beneficial in giving the body a longer time to dispose of the more harmful metabolites. In 1914, Asser (10), working with dogs, found that ethanol decreased the excretion of formate resulting from methanol ingestion.[4] In 1943, Röe (206) concluded that ethanol decreased the toxicity of methanol and delayed the onset of symptoms until after the ethanol had been metabolized. It has since been conclusively shown that ethanol will greatly delay the disappearance of methanol from blood and urine. In rabbits (6) a single dose of ethanol (1.7 gm./kgm.) almost completely prevented a fall in blood methanol concentration for eight hours, that is, until the ethanol had been oxidized. Similarly, in two persons poisoned with methanol, administration of ethanol prevented a drop in blood methanol (7). After ethanol had been withheld for ten or twelve hours the methanol level began to fall once more. The authors concluded that a blood level of 0.1 per cent ethanol is sufficient to block methanol oxidation. Leaf and Zatman (135) gave volunteers 3 to 7 gm. of methanol and measured the concentration of methanol in the urine at different intervals. (They had established the fact that the urinary methanol concentration is 1.3 times the blood level and the urinary concentration is therefore a valid measure of methanol in the body.) After a single dose of 15 ml. ethanol added to the methanol the urinary methanol values were almost constant for several hours and then declined ex-

[4] This original observation was not as clear in its implications as might seem. Asser reported that amyl alcohol (0.5 ml./kgm.) and acetone (2.5 ml./kgm.) reduced formate excretion almost as effectively as ethanol (2.5 ml./kgm.). Also ethanol, amyl alcohol and acetone each reduced formate excretion after sodium formate administration, *i.e.*, these agents seemed to accelerate formate oxidation rather than inhibit its formation.

ponentially as in the controls. If 10 ml. ethanol were given every hour the rate of reduction in urinary methanol was only 10 or 15 per cent of the controls. Similar results were obtained by Kendal and Ramanathan (118).

The original observation of Asser that ethanol will inhibit formate excretion after methanol ingestion has been confirmed. Bastrup (21) found that ethanol almost completely prevented formate excretion following methanol administration in a rabbit and reduced formate excretion by 40 per cent in a dog. Kendal and Ramanathan (117) studied human volunteers who received 4 ml. methanol with or without a supplement of 10 ml. ethanol per hour. With methanol alone, formate and methanol excretion paralleled each other. With ethanol supplementation, formate disappeared completely from the urine; methanol excretion continued. Smaller doses of ethanol were not very effective in inhibiting either formate excretion or methanol disappearance.

Using C^{14} tagged methanol in the rat, Bartlett (18) found that complete oxidation to CO_2 could be inhibited 90 per cent with 1 gm. of ethanol per kgm. every 4 hours. With rat liver slices the oxidation of 0.03 per cent methanol to CO_2 was inhibited 72 per cent and 95 per cent by 0.04 per cent and 0.1 per cent ethanol, respectively.

Lutwak-Mann in 1938 (144) succeeded in partially purifying alcohol dehydrogenase from horse liver and found that it oxidized methanol about one seventh as rapidly as ethanol. Zatman (249), using a similar enzyme preparation, observed that equimolar amounts of ethanol completely blocked methanol oxidation. There was some inhibition with a one to sixteen ratio of ethanol to methanol and the inhibition was "found to be competitive in type".

These *in vitro* studies would seem to explain completely the *in vivo* findings in regard to the relative rates of disappearance of methanol and ethanol and the inhibition of methanol metabolism by ethanol. That is, (a) ethanol and methanol are oxidized by the same enzyme, but at differing rates, and (b) ethanol has a smaller Michaelis constant for the enzyme, hence as long as ethanol is present it monopolizes the enzyme. Unfortunately, a difficulty with this explanation soon arose. Alcohol dehydrogenase was crystallized by Bonnichsen and Wassén in 1948 (30) from horse liver. Theorell and Bonnichsen (226) reported that this pure enzyme would not react with methanol "to any extent". This forced an attempt to find another mechanism for the oxidation of methanol. Keilin and Hartree (115) had found that catalase with H_2O_2 is capable of oxidizing methanol as well as ethanol (114) and had suggested that catalase was important in the metabolism of alcohols in the body. Chance (41) calculated that there is sufficient catalase in liver to account for the rate of methanol disappearance *in vivo*. Jacobsen (106) therefore suggested that catalase is responsible for the oxidation of all the methanol and one-fifth of the ethanol metabolized in the body, whereas alcohol dehydrogenase oxidizes the remaining four-fifths of the ethanol. Bartlett (19) pointed out the difficulty with this explanation, which is that catalase oxidizes methanol and ethanol at about the same rate and with the same kinetic constants, so ethanol would not be a very effective inhibitor of methanol oxidation.

Strittmatter (222) found that a crude liver preparation washed free of substrates would not catalyze the oxidation of methanol by DPN but would readily oxidize ethanol. This preparation would, however, oxidize methanol if a source of peroxide was added to the system. He concludes that methanol oxidation in the body is accomplished either by catalase or by some unknown mechanism. He points out that the experiments of Lutwak-Mann and of Zatman were carried out with methylene blue as the oxidant and this would result in peroxide formation.

It is therefore necessary to say that the mechanism of methanol oxidation and its inhibition by ethanol is not yet settled.

V. GENERAL ANESTHETICS, HYPNOTICS, SEDATIVES

There is no particular reason why all substances which depress the central nervous system must act by a single mechanism. Nevertheless, the premise that this is so has greatly influenced investigations of these drugs. For many years research emphasis has been on attempts to discover possible inhibition of oxidative enzyme systems. More recently, it has shifted to the measurement of the effects of depressants[5] on the generation and utilization of ATP. The older studies have been reviewed by Butler (38) and Quastel (193, 194, 195). The present discussion will be limited to a review of the more crucial data on certain fundamental points and will emphasize information gained in the last five years.

1. Action on glycolytic enzymes. Substances such as chloretone, chloral hydrate, tribromethanol, phenobarbital, pentobarbital, and amytal have very little effect on anaerobic glycolysis at low or anesthetic concentrations (170, 243). Somewhat higher concentrations (high enough to produce 20 to 50 per cent inhibition of respiration) cause little change in anaerobic glycolysis but greatly stimulate aerobic glycolysis. Very high drug concentrations decrease both anaerobic glycolysis and the stimulated aerobic glycolysis. It must be concluded that these agents, at therapeutic concentrations, are without direct effect on glycolytic enzymes. This conclusion gains further support from the experiments of McIlwain and coworkers (Section V, 4). On the basis of older interpretations the increased aerobic glycolysis would be considered secondary to inhibition of oxygen uptake. In some cases we must now raise the possibility of uncoupling of phosphorylation as a cause of the increased glycolytic rate. This will be discussed later.

2. Action of depressants on the respiration of unstimulated tissue and on oxidative enzymes. One theory for the mechanism of action of anesthetics and related drugs is that these agents depress nervous tissue by inhibition of its respiration. It is a natural expectation that depression of function would result from depression of metabolism. The oxygen consumption of brain *in vivo* is, in fact, decreased during anesthesia (80, 95, 96, 119). The difficulty is to distinguish cause from effect. That is, does the depression of function result in a lessened energy expenditure and lowered oxygen use or *vice versa*?

Many depressant drugs have been shown to inhibit respiration in isolated

[5] In this section the term depressant is used to indicate substances which have a general depressant action on the central nervous system.

tissue preparations. Jowett and Quastel (112) and Jowett (111) reported a 6 to 32 per cent inhibition of the respiration of brain cortex slices by concentrations of barbiturates, chloral hydrate, tribromethanol, ethylurethane, and chloretone, corresponding roughly to the anesthetic doses in animals. However, much of the early work was carried out with concentrations considerably higher than ever likely to be present *in vivo*. Furthermore, many depressant drugs, for example ether (113), alcohol (36, 65), and a number of barbiturates (59, 64), do not inhibit respiration of brain slices at therapeutic concentrations.

Westfall (244), investigating the effect of phenobarbital on cortex slices over a range of concentrations that would correspond to sedative and hypnotic as well as anesthetic doses, found that low concentrations (10^{-5} M) actually produce 10 per cent stimulation of respiration. It required 10^{-3} M, or roughly ten times the anesthetic level, to produce significant inhibition. Similar results have been obtained with other barbiturates (245, 246). Gerard (74) also has reported that some barbiturates which are good functional depressants *in vivo* stimulate oxygen consumption of brain tissue *in vitro*, while others which are poor anesthetics markedly inhibit respiration. Moreover, Larrabee *et al.* (130, 131, 132, 133) have shown that a variety of depressant drugs block synaptic transmission in sympathetic ganglia at concentrations lower than those which decrease oxygen uptake.

Most workers in the field, including Quastel, now agree fairly well that any depression of the overall oxygen consumption of unstimulated brain tissue by depressants at anesthetic levels is small, if not completely absent. However, Schueler and Gross (210) have reported inhibition by blood from anesthetized animals. Quastel's most recent view (195, 195a) has been that anesthetics selectively depress a sensitive oxidative reaction vital to the generation of ATP. Green (76), Quastel (193, 193a, 194, 195) and others (119) have repeatedly emphasized that there might be much greater inhibition in local areas most sensitive to the drugs. Such inhibition might not be detectable in measurements of overall oxygen consumption. This possibility is of course difficult to prove or disprove, and there is understandable reluctance to give up such a reasonable theory, but there can hardly be said to be much supporting evidence for it. The most direct evidence suggesting that there are certain sensitive areas related to the phenomenon of anesthesia are the experiments of Magoun (147) on his "reticular activating system." Very low concentrations of ether and pentobarbital stop conduction here without effect on other areas. This system is more sensitive to cyanide than other brain areas (147a, 147b), but it has not been shown that the respiration of this area is particularly sensitive to inhibition by anesthetics.

Very high concentrations of depressants do inhibit some of the dehydrogenases involved in glycolysis (inhibition of anaerobic glycolysis) and in the citric acid cycle (inhibition of oxidation with a dye as electron acceptor). This is apparent in some of the older experiments of Quastel, and more recently has been observed with 4×10^{-3} M pentobarbital by Persky *et al.* (187). These effects probably have no significance in anesthetic action, for the anesthetic concentration of pentobarbital is no more than 1 or 2×10^{-4} M. From the point of view of mechanism of an enzyme inhibitor it is of interest that pyruvate and high concentrations

of pentobarbital appear to compete for the same site in the pyruvic oxidase system and that BAL blocks inhibition by pentobarbital (187). Earlier Michaelis and Quastel (170) noted competition between lactate and certain anesthetics for lactic dehydrogenase.

An interesting point which came out of the early work with moderately high concentrations of anesthetic agents was the demonstration that those anesthetics which depress respiration appear to do so by inhibiting a particularly sensitive point in the system for transferring electrons from substrate to oxygen (193, 194). For example, barbiturates, ether, and chloretone in concentrations which strongly inhibit oxidation of glucose[6], lactate, pyruvate, and glutamate have little effect on the oxidation of succinate or p-phenylenediamine (112, 196). No inhibition with p-phenylenediamine clearly indicates lack of effect on the cytochrome oxidase segment, a point which has been confirmed by many workers. No inhibition of succinoxidase indicates, in addition, that electron transfer from succinic dehydrogenase—a flavoprotein—via cytochrome *B* to cytochrome *C* is also unaffected. Michaelis and Quastel (170) found that passage of electrons from substrates to flavoproteins, to various dyes via flavoproteins, or to ferricyanide was only slightly inhibited by chloretone. Thus it appeared that depressant drugs were interrupting a link between these particular flavoproteins and cytochromes. Chloretone inhibited the transfer of electrons from DPN to oxygen by the flavoprotein-cytochrome *C*-cytochrome oxidase system in a Keilin and Hartree preparation from pig heart.

Greig (78), working directly with brain homogenates, confirmed the basic findings of Quastel's group in demonstrating that pentobarbital, chloroform, and chloretone inhibit normal respiration but do not inhibit if the electron transfer chain above flavoprotein is by-passed with methylene blue. Similar results were obtained with lactate oxidation by yeast. Ernster *et al.* (56a) have restudied the site of barbiturate inhibition of respiration and firmly established the suggestions of earlier workers.

The nature of the interaction between the flavoproteins, which accept electrons from DPNH, and the cytochromes, which reoxidize the flavoproteins, is still not entirely clear. There have been repeated suggestions that one or more intermediate links are involved (190, 214). The partial purification of what seem to be single enzymes catalyzing the reduction of cytochrome *C* by DPNH or TPNH (83, 100, 149) introduced some doubt about the existence of such a link. Extensive studies of the effects of depressant substances on these partially purified enzymes have not been made, but several isolated negative reports are available. Persky *et al.* (187) and Kohlenbrenner (see Brody and Bain, 32) have reported that DPNH-cytochrome *C* reductase is not especially sensitive to depressant drugs.

Persky *et al.* (187) conclude that the lack of effect of pentobarbital on DPNH-cytochrome *C* reductase and on flavoproteins such as D-amino acid oxidase

[6] The greater sensitivity of respiration with glucose as a substrate compared to lactate and pyruvate may well be secondary to uncoupling effects, since glucose must first be phosphorylated by ATP.

definitely refute the hypothesis of an action of barbiturates on flavoprotein oxidation, but this conclusion appears largely unjustified. The flavin enzymes form a large and heterogeneous family with no common denominator except the riboflavin in the prosthetic groups. The failure to find inhibition of the activity of the flavoprotein D-amino acid oxidase, for example, by no means indicates that other flavoproteins may not be inhibited. This amino acid oxidase normally reacts directly with oxygen, in sharp contrast to flavoproteins of the electron transfer chain.

Lack of inhibition of DPNH-cytochrome *C* reductase would seem to leave us without a site of action to explain inhibition of respiration by higher concentrations of anesthetics. However, caution should be used in drawing conclusions from studies with the purified enzyme, for effects on the enzyme in the cell (presumably in the mitochondria and microsomes) may be somewhat different. It has been found that DPNH-cytochrome *C* reductase in the tissue is inhibited by antimycin A, but the partially purified enzyme is not (52). Recent evidence has been presented (148) to show that even the reaction catalyzed by the isolated enzyme consists of several steps. Ernster *et al.* (56a) have discussed the studies of earlier workers which indicate two pathways for electron transport in mitochondria, only one being sensitive to antimycin *A*, and point out that a similar picture holds for amytal as an inhibitor. Moreover, it must be kept in mind that if the DPNH-cytochrome *C* reductase isolated is the principal enzyme responsible for oxidation of DPNH in the cell, its function in the cell is so closely coupled with phosphorylation that lack of phosphate acceptors will result in no transfer of electrons. Thus any substance interfering with normal reaction of phosphate or phosphate acceptors with the enzyme would inhibit respiration as well as interfere with phosphorylation. In this manner an inhibitor of an enzymatic step not directly concerned with the passage of electrons from DPNH to cytochrome *C* might nevertheless block the passage of those electrons in the intact integrated enzyme matrix of the cell.

3. Action on phosphorylation coupled to oxidation. The accumulation of data indicating that anesthetic and hypnotic agents caused either no change or slight inhibition of respiration at concentrations which are anesthetic *in vivo* and the suggestion that they may actually stimulate respiration slightly at sedative levels led to speculation that they might interfere with the energy available for cellular processes in some other way, perhaps by interfering with ATP formation or utilization (see McElroy, 158). In 1950, Butler (38) discussed the possibility that limitation in the use or rate of hydrolysis of ATP might cause a decrease in oxygen consumption. This was two years before the demonstration that oxidation may virtually stop in mitochondria when no acceptors for high energy phosphate are available (129a).

The direct study of the effect of various anesthetic and hypnotic drugs on generation of ATP has been largely carried forward by Brody and Bain and coworkers. The background and results of their early work with barbiturates have been reviewed by Bain (12) and by Brody (30c).

Brody and Bain (32, 33) first observed that some barbiturates, particularly

thiopental, have a selective effect in decreasing ATP formation more than oxygen consumption in brain and liver mitochondria. In fact, very low concentrations of thiopental (too low to affect phosphorylation demonstrably) stimulate oxygen uptake slightly. Thus, these substances behave much like uncoupling agents such as dinitrophenol (DNP), although concomitant increase in oxygen consumption and decrease in P:O ratio have been hard to demonstrate. Like other uncoupling agents, barbiturates increase the apparent ATPase of mitochondria (14, 156). One important feature of these effects is that they are produced by concentrations of thiopental which, on the basis of the anesthetic dose, might be expected to occur *in vivo*. Another significant feature is that the effect can be reversed to some extent by washing out the drug.

The uncoupling phenomenon is not limited to the brain alone or to the barbiturates. Abood *et al.* (2) noted some decrease in phosphorylation in spinal cord mitochondria. More recently Bain (13, 15) has reported that chloral hydrate, methadone, and even salicylates have uncoupling action. Hulme and Krantz (104) have reported that ether in surgical concentrations causes 20 per cent uncoupling in a particulate preparation from rat brain.

These observations provided direct support for the theory that anesthetics and other depressants may act by decreasing the amount of ATP available for cellular functions. However, uncoupling of phosphorylation cannot be applied as a general explanation of narcosis, for Bain (13) finds that chlorobutanol, often used as a typical "narcotic" in the older literature, does not uncouple. Negative findings have also been reported for morphine (13), nitrous oxide, and xenon (136). These results would not necessarily invalidate the uncoupling hypothesis as a mechanism of action for compounds in the barbituric acid series. However, when Brody and Bain (33) extended their studies, they found that some barbiturates, potent as depressants, have little or no uncoupling action. Although simple uncoupling cannot be the whole explanation for depressant actions, the phosphorylation experiments merit close scrutiny for a full understanding of barbiturate action.

Concentrations of barbiturates which produce uncoupling depress oxygen consumption.[7] When the depression of oxygen consumption relative to uncoupling is examined closely (Table IV), it is seen that none of the barbiturates produces as "pure" an uncoupling as DNP.[8] The thiobarbiturates show the greatest selectivity in uncoupling phosphorylation. Butethal and hexobarbital produce very little uncoupling below 7.5×10^{-4} M. Diallylbarbital and phenobarbital appear to exert no uncoupling action at all. The uncoupling and the depression of oxygen consumption do not appear to be correlated in any obvious fashion with the *in vivo* anesthetic potency of the barbiturates. The convulsant

[7] Eiler and McEwen (54) found that depression of oxidation and phosphorylation were nearly equal.

[8] Depression of oxygen consumption is usually not seen with uncoupling concentrations of DNP. However, Brody and Bain (33) found that with brain mitochondria there was a small inhibition by DNP. The depression of oxygen consumption by thiopental is less with liver mitochondria.

TABLE IV

Effect of barbiturates on oxidation and phosphorylation

(From the data of Brody and Bain, 33)

Drug	Approximate drug concentration required for 50 per cent decrease in P:O in brain mitochondria	Per cent depression of ΔO_2 by this drug concentration
DNP	1×10^{-5} M	10
Thiohexethal	2.5×10^{-4}	30
Thioethamyl	4×10^{-4}	30
Thiopental	4×10^{-4}	30
Spirobarbital®	4×10^{-4}	35
Thiamylyl	$3(?) \times 10^{-4}$	40
Pentobarbital	3×10^{-4}	50
Amobarbital	3.5×10^{-4}	50
Convulsant barbiturate*	$1.5(?) \times 10^{-4}$	ca. 50
Secobarbital	$2(?) \times 10^{-4}$	ca. 65

* Convulsant barbiturate = 1,3-dimethylbutylethyl barbiturate (224).

barbiturate listed in Table IV inhibits oxygen consumption in low concentrations, but it is not a highly selective uncoupling agent.

Ability to penetrate the brain appears to vary a great deal among the barbiturates. There may be a barrier at the mitochondrial surface as well, which is not penetrated equally by the different drugs tested during the fifteen to thirty minute experimental period. This might conceivably explain some of the differences in activity of various barbiturates on brain mitochondria. The thiobarbiturates, which act rapidly, are more soluble in fats and show the greatest selectivity in uncoupling phosphorylation. Diallylbarbiturate and phenobarbital act slowly and show no uncoupling.

The degree of uncoupling by barbiturates varies with the substrate in a somewhat puzzling manner. Thiopental at 8×10^{-4} M completely uncoupled with pyruvate + malate as substrate, yet 1×10^{-3} M produced only 70 per cent uncoupling with succinate. The oxygen consumption was depressed less with succinate (see also 54, 56a).

It is clear that there are a number of differences between the action of barbiturates and typical uncoupling agents such as DNP. Not only is the uncoupling less selective and more limited in action than with DNP, but even the most selective barbiturate, thiopental, has much less effect than DNP in stimulating oxygen uptake in low phosphate systems. Since with barbiturates it is not possible to achieve a high degree of uncoupling without serious depression of oxygen uptake, it is difficult to be certain that barbiturates uncouple all of the phosphorylations affected by DNP. It is distinctly possible that phosphorylation due to succinate oxidation, for example, is only partially uncoupled by thiopental, whereas DNP uncouples completely. Ernster *et al.* (56a) have reported that amytal does not uncouple phosphorylation at all with succinate. Johnson and Quastel have reached similar conclusions for chloretone (109a).

The very fact that uncoupling by anesthetic agents is less complete than with DNP raises the possibility that the action of these agents may be restricted to certain sites of ATP generation. The concept of coupling of ATP, generated at a specific point, to some specific reaction seems to be implied in the view of Johnson and Quastel (109, 193a). Such a concept has not received much attention, as biochemists have preferred to think of all ATP entering a common pool. However, ideas about mixing of intermediates with common pools have been drastically revised in recent years.

Several facts raise the question whether the effects of barbiturates, ether, and other anesthetic agents—on mitochondria at least—might be due to changes in structural organization resulting from affinity of these agents for phospholipids in mitochondria. The evidence may be summarized as follows. The more fat soluble thiobarbiturates are the best uncoupling agents. The phospholipids have been suggested as being vital in the organization of integrated enzyme systems (16). Alteration in mitochondrial structure usually results in loss of phosphorylation with less effect on electron transfer. Irreversibility of effects with higher concentrations of anesthetics, such as observed for ether by Quastel and Wheatley (196, 197), could be due either to direct damage or a secondary decline from lack of ATP. More limited reversibility of barbiturate effects in brain than in liver mitochondria might be due to more lipid in brain preparations (33) or to inherent lower stability of the enzymes.

It is of considerable interest that the apparent site of inhibition of oxygen consumption by higher concentrations of narcotics (DPNH-cytochrome *C* reductase system as it exists in the cell and isolated particulate elements) involves an enzyme system which probably has two phosphorylations coupled to its action. Greig (79) has also taken note of this interesting fact. DNP frees this system from any limitations normally imposed by phosphate acceptor requirements but does not inhibit respiration. The action of the barbiturates on this system, obviously somewhat different from that of DNP, may not only prevent phosphorylation but also have a considerable tendency to inhibit electron transfer.

The objections to the uncoupling theory may be summarized as follows. First, DNP is not an anesthetic agent, although it apparently can increase the effect of barbiturates under special conditions (12, 13, 34a). Although Bain (13) has raised the possibility that DNP may have less effect than expected on the brain *in vivo* because of failure to penetrate, this possibility seems unlikely on the basis of general experience with DNP and its lipid solubility. However, further work needs to be done, for Brody (30c) has pointed out that very little pentachlorphenol appears in the brain. Another obstacle is that fever and increased metabolic rate are not characteristics of barbiturate poisoning, as might be expected if there was much similarity to DNP. A third objection is that some depressant barbiturates show little or no uncoupling action, while 1,3-dimethylbutylethylbarbiturate, which is a stimulant and produces convulsions, does show some uncoupling effect (33). However, the stimulant action of this barbiturate might represent an additional effect overshadowing other effects. It is well known that the nervous system can respond with convulsive discharges during ether and

barbiturate anesthesia. A fourth objection is that hypnotic and sedative doses of barbiturates probably do not produce tissue concentrations sufficiently high to cause uncoupling of phosphorylation. Brody and Bain (33) found that even the most active barbiturates first produce detectable lowering of the P:O ratio at concentrations of 1×10^{-4} M which corresponds roughly to the dose per kgm. for *anesthesia, i.e.*, several times the sedative dose. Although barbiturates are not concentrated to any great extent in the brain as a whole (157), it must be admitted that there is no information available as to the critical *intracellular* concentration to which mitochondria would be exposed. A final objection to the uncoupling theory is that the phosphocreatine level of the brain is not decreased during anesthesia. If anything it is increased (159).

4. Action on respiration and glycolysis in stimulated isolated tissue. Some very interesting information about the effect of drugs on the metabolism of brain tissue *in vitro* has come from the extensive experiments of McIlwain and coworkers (37, 39, 62, 137, 164, 165, 205). The results of their studies have led them to conclude that brain slices are not equivalent to intact nervous tissue since there is a complete absence of stimulation from neighboring nervous elements.

The oxygen consumption and glycolysis of brain slices are both less than that of brain *in situ*. When, however, brain slices are subjected to electrical stimulation, the glycolysis and oxygen consumption are increased to a level approximating that of intact brain. The general characteristics of the electrical stimulus required to produce these increases are such (11, 62, 160) as to make it seem likely that nerve cells in the slice respond with increased electrical activity and that a situation is induced which resembles active nervous tissue.

McIlwain's group confirmed earlier work, showing that the oxygen consumption of unstimulated slices was little affected by low concentrations of depressant drugs. However, when the oxygen consumption was increased 50 to 100 per cent by electrical stimulation, it was more sensitive to drug inhibition. (See also Bronk and Brink, 35.) In the case of general depressants there was some inhibition by concentrations approaching those expected *in vivo*, with much greater inhibition by higher concentrations (39, 164). This suggested that the increased oxygen uptake due to activity in nervous tissue was sensitive to drugs, while the resting oxygen consumption was not. Unstimulated cerebral cortex slices maintain phosphocreatine levels near *in vivo* values (94, 167). Since phosphocreatine falls to low levels in electrically stimulated slices, McIlwain and coworkers (37, 94, 159, 162, 163, 164) have used the working hypothesis that the increased respiration is due to increased cellular activity, *i.e.*, to increased utilization of energy-rich phosphate compounds. Stimulation of ATPase would produce the same result, but is a less likely possibility. While there might be some decrease in ATP synthesis during convulsions, as suggested by Abood and Gerard (3), it seems unlikely that this is the major effect.

The ability of general depressants, such as barbiturates and chloral, to reduce electrically stimulated oxygen consumption might be the indirect result of a decreased neuronal response to electrical stimuli or a direct result of inhibition of an enzyme in the oxidative system. Considerable information on this point

has been obtained by studying the effects of depressants in the presence of substances which increase the metabolism of brain slices (164, 169). It was found that the respiration of slices of cerebral cortex with glucose, lactate, or pyruvate as substrate was increased by (a) electrical stimulation of appropriate characteristics, (b) DNP, (c) high K^+ in the medium, and (d) low Ca^{++} in the medium. Moreover, depressants, such as barbiturates and chloral, inhibited the *stimulated* respiration *in all cases* (164, 169). Quastel (193a) has confirmed some of these experiments, demonstrating significant inhibition with concentrations of barbiturates and alcohol likely to be present *in vivo*. This suggests that in the passage of electrons to oxygen in the stimulated slices the rate limiting reaction is sensitive to these depressants. This reaction apparently is not the rate limiting step in unstimulated tissue (164). An examination of the mechanism by which the various substances stimulate respiration should provide some clues as to rate limiting reaction in the unstimulated and in the stimulated situation.

The first clue lies in the action of DNP. Although the intimate mechanisms are not known, it is recognized that DNP in general frees the consumption of oxygen from limitations imposed by lack of phosphate acceptors. In result its action would be similar to increased ATP utilization by increased neuronal activity (163). The stimulation of respiration by DNP suggests that a major limiting factor in rate of respiration in resting tissue is unavailability of phosphate acceptors due to accumulation of ATP and phosphocreatine. This is consistent with the observation (94, 167) that slices build up phosphocreatine levels approaching those *in vivo*. McIlwain and Heald (94, 163) found that low Ca^{++} or high K^+ caused a marked drop in the phosphocreatine levels in slices, just as was seen with electrical stimulation and DNP. The mechanism by which low Ca^{++} or high K^+ produces these changes has not been clearly defined, but the evidence suggests that the different stimulating procedures used all increase respiration by increasing availability of phosphate acceptors.

With the reactions in electron transfer relieved from the limitation imposed by a shortage of phosphate acceptors, the respiration increases 50 to 100 per cent and now a reaction inhibited by various depressant drugs can become rate limiting. Since studies with higher concentrations of depressant drugs (78, 170) suggested inhibition between flavoproteins and cytochrome *C*, this seems the most likely point for inhibition of stimulated respiration by somewhat lower concentrations of the drugs. For example, in resting tissue where phosphate acceptors may be the limiting factor, a 50 per cent reduction in active enzyme in the DPNH-cytochrome *C* reductase region would produce little effect on the overall respiration if this were not the rate limiting step. However, with the rate of respiration doubled, this step may become the limiting one and even 10 per cent inhibition (lower concentrations of drugs) would now be detectable.

Additional information from the experiments of McIlwain and coworkers may be examined for consistency with the interpretation given above. With fairly high concentrations of succinate as substrate the oxygen uptake is high (more like the stimulated rate with glucose or pyruvate), the respiration is not stimulated by electrical impulses, and the phosphocreatine values are low (126, 127,

161). However, the respiration is not inhibited by barbiturates. Two facts seem clear. First, electrical stimulation does not increase respiration if there are already adequate levels of phosphate acceptors. Second, succinate oxidation, even when freed of possible limitation by lack of phosphate acceptors, is not limited by the electron transfer step sensitive to general depressants. This is in complete agreement with the conclusions of earlier workers.

Some observations appear to raise the question whether the reaction rates increased as a result of stimulation and the point inhibited by depressants are in an electron transfer chain common for all DPN dependent oxidations. For example, glutamate oxidation in rat or guinea pig cortex slices was not increased by electrical stimulation (137, 161). This lack of effect by electrical stimulation on an oxidation involving electron transfer by the same general pathways as glucose and subject to the same phosphate acceptor limitations was puzzling until it was demonstrated (37, 163, 167, 168) that whenever glutamate was added to the tissue the phosphocreatine levels were very low. In brain tissue from monkey or man the results with glutamate were more like those with glucose or pyruvate (166). In most animals the rate of respiration with glutamate is higher than with glucose, but in spite of adequate phosphate acceptors, it is not as high as the stimulated rate with glucose. Transfer of electrons from glutamate to DPN may be the limiting step in this case. Little information is available in the published experiments of McIlwain, but earlier workers reported that glutamate oxidation was sensitive to depressants (193, 194).

Other observations which raise questions about the site of the limiting step are lack of electrical stimulation of oxygen consumption with α-ketoglutarate, citrate, and fumarate as substrates (126, 137, 161, 166) even when the rate of oxygen consumption is no higher than the unstimulated rate with glucose or pyruvate. Since the phosphocreatine level, already low with fumarate or citrate (126), would presumably be lowered still further by electrical impulses, factors other than phosphate acceptors must be limiting when these intermediates, normally present in catalytic amounts, are added to the tissue slices in relatively high concentration. Permeability is a possible limiting factor, but the low phosphocreatine levels, when the oxygen consumption is equal to that with glucose, suggest an abnormal situation resulting from inhibitory effects. McIlwain (126) has shown in the case of succinate that 0.004 M yields very much better phosphocreatine levels than 0.02 M. Low concentrations of depressants would have little effect on respiration with these substrates if other steps are limiting.

Electrical stimulation of slices increases glycolysis to about the same extent as it increases oxygen consumption. If the principal actions of a depressant were inhibition in the electron transfer chain and/or uncoupling of phosphorylation, the stimulated glycolysis which occurs with electrical impulses, DNP, or K^+ should not be inhibited. In fact, with inhibition of oxygen uptake more lactate might accumulate due to decreased removal. Examination of the experiments of McIlwain's group (137, 164) reveals a somewhat complicated picture in this regard. The changes in aerobic glycolysis (lactate accumulation) with various drugs are less consistent than changes in respiration. This situation undoubtedly

results from the complication that lactate accumulation is a balance between formation and removal.

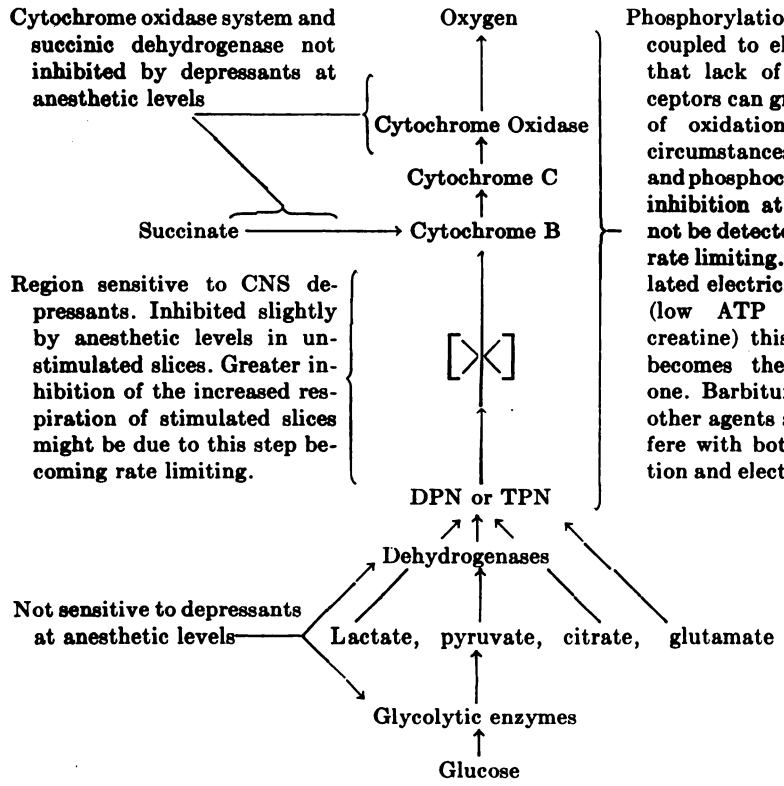
Essentially all general depressants increase aerobic glycolysis in unstimulated slices at concentrations which leave oxygen consumption little affected. Most general depressants also increase aerobic glycolysis in stimulated slices (39, 164), regardless of how the stimulation of metabolism is produced (electrical, DNP, high K^+). Since decreased neuronal activity would be expected to lower both glycolysis and respiration, inhibition of respiration with an actual increase in glycolysis in electrically stimulated slices indicates that the effect on the respiratory enzyme systems must be a direct one. It is interesting that the inhibition of respiration of slices by anticonvulsant drugs (62), in contrast to anesthetic agents, appears to be secondary to decreased neuronal activity.

Abood *et al.* (1, 4, 4a) have reported uncoupling of phosphorylation in mitochondria by electrical impulses. This would suggest that the increase in respiration and glycolysis in McIlwain's experiments might be due to a DNP-like action, not increased neuronal electrical activity. However, Narayanaswami and McIlwain report (176) that electrical stimuli which produce maximal effects on preparations containing whole cells have no effect on isolated mitochondria. They call attention to the fact that since mitochondria are labile systems, they might be damaged by certain types of electrical currents of sufficient intensity (especially when protective factors may have been washed away). Also inhibitory substances appear to be released from many types of electrodes. Abood and Romanchek (4a) have confirmed the fact that uncoupling is seen only with certain types of electrodes. It is seen with all types of electrodes when ferricyanide is added as electron acceptor. The effect appears to be mediated by metal ions and may involve oxidation of an —SH group, as it is blocked by glutathione. The strongest support for a direct action of electrical impulses is that the uncoupling is so quickly reversed on stopping the electrical current. However, this does not absolutely preclude some oxidative chemical change which is quickly reversed in a system metabolizing substrate. The fact that cocaine and atropine block the response to electrical stimulation but not to high K^+ in the cortex slice experiments of McIlwain's group argues against uncoupling by electrical impulses under their conditions. It is unlikely that these drugs would prevent uncoupling due to electrical impulses. Cocaine and the very high concentration of atropine used probably decrease neuronal response to electrical stimulation.

5. Relation of enzyme effects to pharmacological actions. It has been seen that hypnotics may produce a variety of effects on enzyme systems of brain. The observed effects, whether produced by very high drug concentrations or by drug levels close to those present during anesthesia, are all such as to *decrease* the production of high energy phosphate bonds. It is therefore difficult to reconcile any of these actions on enzymes with the fact, affirmed by a number of workers, that the concentrations of energy-rich phosphate compounds in the brain during anesthesia are increased rather than decreased (reviewed by McIlwain (159, 162) and Richter (203)). It seems inescapable that the primary *in vivo* effect of most hypnotics and anesthetics is a decrease in neuronal activity, with decreases

Effect of Hypnotic-Anesthetic Substances on Oxidation and Phosphorylation

It is doubtful that anesthesia is produced primarily through a general depression of oxidation or phosphorylation. Nevertheless, anesthetic agents in higher concentrations, such as those used to induce deep anesthesia, do appear to have direct effects on some enzyme systems. The following scheme may be helpful in understanding these effects.



in oxidation being largely secondary to the decreased utilization of ATP. The decrease in neuronal activity may be the result of special sensitivity to these drugs at synapses in general (30a, 30b, 130) or at synapses of the reticular activating system (147, 147a, 147b). Both light and electron microscope studies indicate greater concentration of mitochondria at synaptic points. Conceivably the metabolism of synaptic tissue or specific regulating nerve centers might be more sensitive to drug depression. This view is favored by Quastel (193a, 195a). It seems equally plausible that specific transmission mechanisms are inhibited by anesthetic agents. It is perhaps the crucial problem in the study of the mechanism of anesthesia to decide between these two possibilities.

VI. PENICILLIN

Although the primary point of action of penicillin is not yet settled, there is evidence that both protein and nucleic acid metabolism are altered. The effects on protein synthesis may be secondary but are nevertheless of interest.

1. Effects on protein synthesis. Chattaway *et al.* (42) found in 1949 that penicillin inhibition of *Corynebacterium diphtheriae* is antagonized by substances of peptide nature in yeast autolysates. This is consonant with the earlier suggestion of Pratt and Dufrenoy (191) that the action of penicillin involves the peptide glutathione. Simmonds and Fruton next reported (213) that a mutant strain of gram negative bacilli, sensitive to penicillin at 1–10 U./ml. when leucine + glycine were provided, was not affected by the antibiotic at 500 U./ml. when these two amino acids were supplied as the preformed peptide leucylglycine.

Thus there is evidence that penicillin may inhibit synthesis of certain peptide bonds. At first it appeared possible that the action of penicilin in causing *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) to lose the ability to accumulate free glutamic acid and certain other amino acids (73) might be implicated in this action, since uptake of amino acids logically would precede peptide bond synthesis. However, inability to concentrate glutamate does not develop until after cells are damaged by growth in media containing 0.1–10 U./ml. of penicillin. Therefore, this is probably a secondary defect. Hotchkiss (102, 103) reported that with *Micrococcus pyogenes* var. *aureus* incubated under conditions which gave minimal multiplication, penicillin (10–30 U./ml.) did not decrease the assimilation of amino acids, but instead of cellular protein synthesis, extracellular peptide-like material accumulated. Gale and Paine (72) have never observed such a direct effect with their strain of organism, but have found peptide accumulation when cells damaged by growth in low concentrations of penicillin (10 U./ml.) are incubated with amino acids. The alterations in this case were indirect (secondary to growth in penicillin) and there were uncertainties about autolysis in these nonviable cells.

In view of the suggestion that penicillin interferes more or less directly with synthesis of certain peptide bonds, the direct action of penicillin on several reactions involving glutathione is of considerable interest. Fischer (60) pointed out the similarity of structure between penicillin and glutathione. Binkley and Olson (28, 120) found that penicillin (1500 U./ml.) competitively inhibits the hydrolysis of glutathione in liver preparations. Even more significant is the observation that penicillin competitively inhibits transpeptidation reactions involving glutathione (90, 91). A general role for transpeptidation reactions in protein synthesis has been proposed, but not yet established. The amounts of penicillin required to inhibit these reactions is very high (6000 U./ml.). As Gale (67) has indicated, if these actions are significant in antibacterial action, the amount of penicillin required to inhibit growth completely would approximate the glutathione concentration of the suspension (or at least equal the glutathione concentration at the vital enzyme). The "competitive" nature of these relationships has not yet been studied in detail, and the possibility of direct reaction between penicillin and GSH should not be ignored, for it would explain why approximately equimolar quantities are required for complete "inhibition" of a reaction. (See 40, 58, 145.) Organisms inhibited by penicillin lose their cytological reaction for —SH groups, and Pratt and Dufrenoy (192) have speculated that this shift in the sulfhydryl-disulfide equilibrium causes a major distortion in the metabolism of the cells.

Recently Gale and Folkes (70) found that, although penicillin in very high concentrations (1000 U./ml.) does not block protein synthesis in *Micrococcus pyogenes* var. *aureus* on glucose and amino acids, low concentrations (0.03–0.3 U./ml.) seem to block an exchange reaction between free glutamate within the cell and glutamate in the protein. It (66, 234) has been reported that penicillin does not interfere with adaptive enzyme formation, but this is certainly not true for β-galactosidase in *Micrococcus pyogenes* var. *aureus* (71a).

Taken altogether, the evidence for a primary effect of penicillin on enzymes involved in peptide bond synthesis is not very persuasive. The high concentrations of penicillin required in most cases to produce peptide actions, the observations of dramatic effects by penicillin on ribonucleic acid metabolism, and the current theories about nucleic acid control of protein synthesis have led to the general feeling that the primary effect of penicillin is in nucleic acid metabolism, with effects on protein synthesis being secondary. This concept received support from the demonstration by Gale and Folkes (67, 68, 69) that protein synthesis is closely correlated with the nucleic acid content of cells and that the stimulation of protein as well as nucleic acid synthesis by purines and pyrimidines is inhibited by penicillin. (The amount of penicillin used in these experiments was low relative to the mass of cells, but high in terms of the units (3000) per ml.) More recently these workers (71a) have shown in disrupted staphylococcal cells that penicillin at 0.2–2.0 U./ml. inhibits protein synthesis only as a result of inhibiting nucleic acid synthesis. More than one hundred times this amount of drug is necessary to give detectable direct inhibition of protein synthesis.

2. Effects on nucleic acid catabolism. In 1947, Krampitz and Werkman (125) studied the effect of penicillin on ribonucleic acid oxidation in *Micrococcus pyogenes* var. *aureus*. These cells were able to oxidize the ribose moiety of either nucleic acid or free pyrimidine nucleotides, but could not oxidize purine nucleotides, nucleosides, ribose-phosphates, or free ribose. Penicillin when used in high concentrations (200–500 U./ml.) was able to block the ribose oxidation both in ribonucleic acid and in free pyrimidine nucleotides. Effects of penicillin on the catabolism of ribonucleic acid derivatives were also discovered by Gros and Macheboeuf (81). They first observed that release of purine bases and phosphate from several nucleotides by non-growing *Cl. sporogenes* was inhibited by penicillin at 500 U./ml. Similarly, under conditions of autolysis, degradation of ribonucleic acid was decreased by penicillin. Since penicillin was reported by Massart *et al.* (155) to block degradation of ribonucleic acid by ribonuclease, early workers (63, 123) interpreted the action of penicillin on ribonucleic acid catabolism as being due to inhibition of this enzyme. However, Gros *et al.* (82) showed that concentrations of penicillin (150–3000 U./ml.) which inhibit ribonucleic acid catabolism do not inhibit ribonuclease. Moreover, the oxidative catabolism of certain *mononucleotides* is also inhibited by penicillin. Dufrenoy and Pratt (51) obtained cytochemical evidence for inhibition of alkaline phosphatase activity and have interpreted the action of penicillin on ribonucleic acid metabolism as being one of preventing dephosphorylation of mononucleotides by alkaline phosphatase.

Opposed to this are the results of Gros and Macheboeuf (81) who found that

high concentrations of penicillin (1000 U./ml.) do not interfere with oxidation of ribose-5-phosphate, but do inhibit oxidation of guanylic acid, guanosine, and uridylic acid in some strains of *Micrococcus pyogenes* var. *aureus* and adenylic acid oxidation in others. They proceeded to show that penicillin inhibits the splitting of the nucleoside link between guanine and ribose-5-phosphate. More recently Gros and Macheboeuf (81) have found that organisms treated with low concentrations of penicillin (2 U./ml.) during the exponential phase of growth have greatly reduced ability to oxidize adenylic acid, guanylic acid, and guanosine (without change in glycolysis or respiration). Thus high concentrations of penicillin produce a direct enzyme inhibition, low concentrations a delayed inhibition which is presumably a secondary consequence. Gros and Macheboeuf conclude that penicillin acts by suppression of a system important in the metabolism (catabolism?) of nucleosides. Taking note of the fact that nucleoside phosphorylases can catalyze either synthesis or cleavage of nucleosides, they include the possibility that penicillin may suppress synthesis of the riboside link. It is to be pointed out that an inhibitory action by penicillin on nucleosidase or nucleoside phosphorylase would explain accumulation of nucleotides and nucleosides in a degradative process only, not in a synthetic one.

3. Effect on nucleic acid anabolism. A number of workers (see review by Gros and Macheboeuf, 81) have shown by both histological techniques and chemical analyses that organisms grown in the presence of penicillin have low ribonucleic acid content compared to the controls, while deoxyribonucleic acid is unchanged. Mitchell and Moyle (173, 174) followed both ribonucleic acid and mononucleotides during various phases of growth with *Micrococcus pyogenes* var. *aureus*. Normally in the early phases of logarithmic growth ribonucleic acid accumulates and purine mononucleotides and nucleosides decrease. In the presence of penicillin there was a small transitory increase in nucleic acid, which was followed by an inhibition resulting in a large accumulation of mononucleotides and nucleosides and a lower ribonucleic acid content of the cells. Later the values approached normal figures. This is an *immediate* effect of penicillin at 2 U./ml.

Park (183) made similar observations on mononucleotide accumulation and has characterized several nucleotides which accumulate in *Micrococcus pyogenes* var. *aureus* grown in the presence of penicillin, 0.5 U./ml. He demonstrated three uridine 5′-pyrophosphate compounds. One derivative contains an N-acetylamino sugar, the other two appear to contain the same structure with the addition of either alanine alone or a peptide of lysine, glutamic acid, and three alanine residues. Similar or identical compounds occur in normal cells, a fact confirmed by Strominger (223), who also found that uridine nucleotides accumulate to five to ten times the normal level in the presence of penicillin in *non-growing* cultures. Strominger found that, normally, C^{14}-labelled uracil is rapidly incorporated into both uridine nucleotides and nucleoprotein. Penicillin inhibits the incorporation into nucleoprotein (see also 71a). Most workers agree that the uridine compounds probably represent normal intermediates, with accumulation occurring when their catabolism or their utilization in nucleic acid synthesis is blocked. However, as Park (184, 185, 186) has pointed out, the occurrence of uridylic acid in combi-

nation with amino acids and peptides may indicate a key role for uracil derivatives in protein synthesis, and might explain why changes in protein metabolism follow penicillin application. The effects of penicillin on the metabolism of uracil compounds may be far from simple, for Hotchkiss (101) has reported an increased disappearance of uracil from the environment when *Micrococcus pyogenes* var. *aureus* is grown in the presence of low concentrations of penicillin.

VII. STREPTOMYCIN

A number of enzymes have been found to be susceptible to inhibition by streptomycin. In fact, so many enzyme reactions are affected by higher or lower streptomycin concentrations as to undermine confidence that any of them represent the primary site of action. Streptomycin is an excellent precipitating agent for certain types of protein and for nucleic acids. This could introduce artifacts into enzyme studies.

In some cases the concentration of antibiotic required for inhibition is only a few fold greater than the minimal antibacterial concentration. For example, there is a marked inhibition of the diamine oxidase activity of various mycobacterial cells by modest amounts of streptomycin (182). Unfortunately we do not at present know the significance of this reaction in bacterial metabolism. Certain of the enzymatic effects reported appear to represent specific actions on certain strains rather than general phenomena. In 1947, Krampitz *et al.* (124) observed inhibition of ribonucleic acid oxidation in a few strains of *Micrococcus pyogenes* var. *aureus*. Others (140) have reported that with *L. arabinosus* and *Torula utilis*, as well as with *Micrococcus pyogenes* var. *aureus*, streptomycin inhibition was relieved by hexose phosphates, while pantothenate restored growth with *Saccharomyces fragilis* (138). However, in other organisms tested these substances do not counteract streptomycin.

Like many other antibiotics, streptomycin (234) inhibits adaptive enzyme formation. This was first established by Fitzgerald and Bernheim (61) for the case of streptomycin inhibition of the oxidation of benzoic acid in mycobacteria. Earlier this was believed to be a direct effect of the antibiotic. It is a good example of one of the pitfalls in this difficult field.

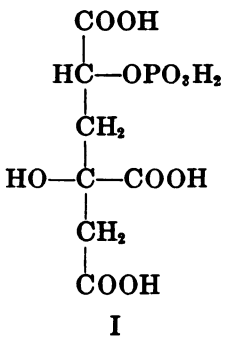
In experiments of Gale and Paine (72) streptomycin inhibited the incorporation of glutamate into cellular substance, but the concentrations required were definitely higher than the minimal amounts for blocking growth. Gale (67) has indicated that low streptomycin concentrations have no effect, but high concentrations do have some inhibitory effect on net protein synthesis in *Micrococcus pyogenes* var. *aureus*. Bernheim (27) has suggested uncoupling of phosphorylation as a possible explanation of inhibition of ammonia assimilation without inhibition of the oxidations normally supplying energy for that process in *Pseudomonas aeruginosa*. However, Gale and Paine found no inhibition of free glutamate accumulation in *Micrococcus pyogenes* var. *aureus*, a process very sensitive to the uncoupling action of DNP.

Of the various enzyme effects of streptomycin that have been reported to date the most convincing as a candidate for the primary site of action is that discov-

ered by Umbreit and coworkers (215, 232, 233, 234, 236, 237). These investigators have made extensive studies of possible enzymatic sites of action of streptomycin in *E. coli*. They found evidence that streptomycin inhibits a reaction involved in the oxidation of a mixture of pyruvate and oxalacetate. There was no inhibition of the conversion of pyruvate to acetate, a substance not further oxidized by cells grown anaerobically under the conditions prescribed. They used streptomycin at 20 microgm./ml., just twice the concentration necessary to block growth, and allowed thirty minutes for penetration into the cells, which they believe is an important limiting step (180, 231).

At first glance it appeared that streptomycin might be inhibiting the enzyme responsible for condensing acetyl CoA and oxalacetate to give citrate. This would explain why there was no inhibition of conversion of pyruvate to acetate. However, it was noted that along with the oxidation of pyruvate and oxalacetate a small amount of citrate was formed. This formation was not inhibited by streptomycin (233, 238). Further investigation revealed that streptomycin does not inhibit purified condensing enzyme or any known reactions of oxalacetate or pyruvate (238). Umbreit and coworkers postulated a second oxidative pathway for pyruvate and oxalacetate that did not involve citrate as an intermediate. They proposed that both the citrate pathway and the hypothetical pathway ("oxalacetate pyruvate reaction") were occurring, the latter being a major pathway in some cases and the only one blocked by streptomycin. The chief evidence for this new pathway for pyruvate metabolism rests on the inhibitory effect of streptomycin (234).

One alternative path would involve condensation of pyruvate and oxalacetate to give a seven carbon intermediate. Exploring the possible seven carbon compounds which might be intermediates, Umbreit (235) found no indication that oxalcitramalic, dihydrooxalcitramalic, or shikimic acid were intermediates. However, when attention was turned to the seven carbon phosphorylated compound, 2-phospho-4-hydroxy-4-carboxy adipic acid, I,



I

which had been isolated from dog liver in 1951 by Rapoport and Wagner (201), some significant findings were made. The possible relationship of this compound to a condensation product between pyruvate and oxalacetate is obvious.

Umbreit found that this new compound appeared in the acid soluble organic

phosphate fraction from bacterial cells as well as animal cells (235). Cells incubated for thirty minutes without substrate had the highest level of the compound. Incubation with pyruvate (with or without streptomycin) resulted in a decrease in the amount of the compound. No P^{32} was incorporated into the phosphate group in either case, although the cells used would have been actively oxidizing pyruvate to acetate.

When fumarate (oxalacetate) plus pyruvate were used as substrate, there was incorporation of P^{32} into the compound, although the absolute level did not increase. When streptomycin was added, there was a slightly higher level of the compound and P^{32} incorporation was greatly inhibited. The reduction in specific activity in the ATP fraction was only 40 per cent, while that in the new compound was 86 per cent. Thus incorporation of P^{32} into the new compound occurs only when pyruvate plus oxalacetate are added and appears to be related to some specific reaction blocked by streptomycin. Much further work will be needed to determine whether this compound is an intermediate, the formation of which is blocked by streptomycin in inhibiting the pyruvate-oxalacetate reaction.

Although Umbreit's general hypothesis involves no inhibition of acetate formation or removal, there are two reports which suggest that formation and removal of two carbon units may be inhibited in some cases. Barkulis (17), with streptomycin at 100 microgm./ml., found that the conversion of pyruvate to acetate plus formate by *E. coli* was inhibited about 75 per cent. Oginsky *et al.* (179) observed that oxidation of higher fatty acids, or more particularly some of the intermediates formed from them, by *Mycobacterium tuberculosis* (Avian) was inhibited by streptomycin. As Umbreit has pointed out, these reactions may involve steps closely related to the oxalacetate-pyruvate condensation.

Umbreit and Tonhazy (239) have presented evidence that the oxalacetate-pyruvate reaction occurs in animal tissues, where it is also sensitive to streptomycin. The mammalian and the microbial enzymes do not differ markedly in their sensitivity to streptomycin. The relatively greater effect on the microorganism than on the host appears to be due to the fact that two barriers, the cell wall and the mitochondrial membrane, ordinarily prevent the antibiotic from reaching the mammalian enzyme in significant amounts. This seems to be a striking example of a case in which permeability factors, rather than inherent difference in sensitivity of enzymes, account for a differential action on parasite and host.

VIII. CHLORAMPHENICOL

Woolley (248) pointed out the structural similarity between chloramphenicol and phenylalanine and suggested that the drug might act to inhibit incorporation of phenylalanine into proteins. He found that phenylalanine can, in fact, counteract inhibition of growth of *E. coli* and *L. casei* by minimal concentrations of chloramphenicol. However, the antagonism by phenylalanine is non-competitive. With *E. coli*, tyrosine and tryptophane had a similar effect, which Woolley attributed to conversion to phenylalanine by this organism.

Woolley's suggestion was borne out by studies with C^{14}-labelled phenylalanine (71). Chloramphenicol inhibited the incorporation of phenylalanine into the protein of *Micrococcus pyogenes* var. *aureus*, but the inhibition was noncompetitive, and chlortetracycline and oxytetracycline had similar effects. Also, the incorporation of proline, leucine, or tyrosine was just as sensitive to chloramphenicol, although the incorporation of glutamate was not. The incorporation of an amino acid into a protein does not prove that protein synthesis has occurred. In the case of both phenylalanine and glutamate, at least, the incorporation is the result of both exchange reactions and net protein synthesis. Glutamate may be more liable than other amino acids to undergo direct exchange reactions with proteins. It seems necessary to conclude that chloramphenicol does not act solely as a structural antagonist to phenylalanine.

Truhaut *et al.* (228) using *E. typhi*, and Bergmann and Sicher (24) with *E. coli*, have confirmed the observation of Woolley that tryptophane has a limited ability to counteract chloramphenicol inhibition in some organisms. Bergmann and Sicher have suggested that chloramphenicol inhibits the sequence anthranilic acid → indole → tryptophane, since growth occurs when indole or tryptophane are supplied but not when anthranilic acid is substituted.

Swenseid *et al.* (225) have reported that chloramphenicol (and aminopterin) inhibition of the growth of *L. citrovorum* is reversed by a substance formed on incubation of folic acid with bone marrow or leukocytes. The inhibition by chloramphenicol is not at the same site as aminopterin, since folinic acid readily reverses aminopterin inhibition but not chloramphenicol inhibition. Folic acid derivatives are involved in many reactions, including most one carbon additions or transfers. Therefore, an action of chloramphenicol on such key reactions could produce changes in both protein and nucleic acid synthesis.

That the most important action of chloramphenicol is a specific one is supported by the experiments of Gale (67, 71), who found with *Micrococcus pyogenes* var. *aureus* that chloramphenicol inhibits protein synthesis without effect on glucose fermentation, ability to concentrate glutamate, extra-cellular peptide accumulation, or nucleic acid synthesis. In fact, nucleic acid synthesis may be doubled when protein synthesis is blocked completely. Tryptophane was included in the amino acids supplied in Gale's experiments. Recently Hopps *et al.* (99) have confirmed these general conclusions in showing that, in *E. coli*, NH_3 assimilation and protein synthesis are strongly inhibited when there is no effect on glycolytic or oxidative phosphorylation and no inhibition of nucleic acid synthesis. Similarly, in *N. perflorens*, polysaccharide synthesis was not inhibited by chloramphenicol.

It seems clear then that, although chloramphenicol does not inhibit the formation of all peptide bonds, it can prevent the formation of the complete protein molecule. Under some conditions this could result from a block in the synthesis of a specific essential amino acid such as tryptophane, but the experiments where 19 amino acids were supplied (Gale) suggest inhibition of the formation of certain peptide bonds essential for completion of the protein molecule.

There are two effects of chloramphenicol on enzymes that are difficult to fit

into this picture because the use to the cell of the enzymes concerned has not been discovered (Umbreit, 236). 1) Owen *et al.* (182), using intact mycobacteria, have observed marked inhibition of diamine oxidase activity by chloramphenicol concentrations in the antibacterial range. 2) Both bacterial esterases (whole cells) and crystalline liver esterase are inhibited by chloramphenicol at concentrations only ten times greater than those which block bacterial growth (216). The observation that chloramphenicol first stimulates and then depresses the bacterial esterases as the concentration is raised is consistent with the fact that very low concentrations of antibiotics may show some stimulation of the growth of microorganisms. The crystalline horse liver esterase behaved very much like the bacterial esterase.

IX. TETRACYCLINES

In 1950, Loomis (141) observed that chlortetracycline was capable of uncoupling aerobic phosphorylation in liver and kidney preparations. Brody and Bain (31) confirmed this observation but did not find uncoupling with oxytetracycline. More recently (34) they have shown that oxytetracycline and tetracycline differ only quantitatively from chlortetracycline. Van Meter *et al.* (240, 241) noted that chlortetracycline inhibition of oxygen consumption, particularly in aged mitochondria, was reversed by Mg^{++}, the first suggestion that the action of these drugs might be related to metal binding. Since then Albert (8) has studied in greater detail the avidity of oxytetracycline and chlortetracycline for a number of metals, but unfortunately did not study Mg^{++}. Brody and Bain (34) have shown that the uncoupling action of all three tetracycline derivatives is prevented if sufficient Mg^{++} is included in the medium. This observation and the fact that these substances do not stimulate respiration in low phosphate systems indicates that their mechanism of action is quite different from that of dinitrophenol, although increased ATP splitting has been found to occur with chlortetracycline (241) as it does with DNP.

Brody and Bain (34) point out that Mg^{++} might counteract uncoupling by 1) removal of chlortetracycline from its site of action by formation of a slightly dissociated Mg^{++} complex, or 2) by replacing essential Mg^{++} which has been removed by reaction with the antibiotic. These workers feel that the available data do not permit a decision between these possibilities, although the second one appears more attractive. However, it should be pointed out that the ratio of Mg^{++} to drug for preventing respiratory inhibition (241) and uncoupling (34) is from 10:1 to 50:1. Even if the tetracyclines complexed several Mg^{++} ions per molecule (and there is no evidence for more than one) there would still be ample Mg^{++} for the enzymes of oxidative phosphorylation. It seems unlikely that added Mg^{++} fails to penetrate into the mitochondria in the presence of the drug. Therefore, the tetracyclines cannot be uncoupling by *removing* Mg^{++}. Added Mg^{++} does protect mitochondria from damage and prevents swelling, and very high concentrations appear to cause shrinkage, so there is a possibility that tetracyclines penetrate poorly into mitochondria suspended in a high Mg^{++} medium.

Actually the concentration-effect curves of Brody and Bain would appear to

fit fairly well with their first suggestion, *i.e.*, that Mg^{++} binds the tetracycline and prevents it from acting. No figure is available for the dissociation constants of Mg-tetracycline complexes, but they are probably more dissociable than the Mn-chlortetracycline complex for which Albert (8) gives a value of 5×10^{-5}. If the figure for Mg-chlortetracycline were 5×10^{-4}, about 80 per cent of the drug would be bound at 2×10^{-3} M Mg^{++}, 95 per cent at 1×10^{-2} M Mg^{++}. This would explain why such high Mg^{++} concentrations are necessary to prevent the effect of tetracyclines. (Actually the picture is more complicated than this would indicate because two tetracyclines appear to complex with one Mg^{++} at low Mg^{++} concentrations.)

There is a third possibility, that tetracyclines uncouple by interaction with Mg^{++} bound to the enzyme without actually removing it. This is essentially the same as saying that the Mg-tetracycline complex is the uncoupling agent. If this were true, the high Mg:drug ratios required for protection would indicate that the Mg-tetracycline complex has a much greater affinity for the enzyme than Mg^{++} alone. It is of interest that in the case of 8-hydroxyquinoline there is evidence that the agent toxic to bacteria is the complex with Fe^{+++} or Cu^{++}, not the free chelating agent (8).

In view of recent work, indicating that Mn^{++} is at least ten times as active as Mg^{++} in protecting mitochondria from damaging agents (105) and that it may be an essential ion for phosphorylation (139), the uncoupling of tetracyclines should be restudied with Mn^{++}. If Mn^{++} is essential in phosphorylation, it acts in concentrations equivalent to or lower than the uncoupling concentrations of tetracyclines. If this Mn^{++} were removed by tetracyclines, restoration of phosphorylation might be possible with Mg^{++}, but only at much higher concentrations. Saz and Slie (208, 209) have presented evidence that Mn^{++} is essential for reduction of DPN by malate in certain *E. coli* extracts and suggest that the inhibition of nitro-reductase by chlortetracycline in such preparations is due to complexing with Mn^{++} and preventing the formation of DPNH essential for reduction. There is a similarity to the case of Mg^{++} and uncoupling in liver mitochondria in that a 50:1 ratio of Mn^{++} to chlortetracycline did not completely prevent antibiotic inhibition of the nitro-reductase.

It is of considerable interest that several workers have observed that Mg ions can neutralize the antibiotic effects of tetracyclines in bacterial cultures (217, 243a). For this effect Sonicin (217) used ratios of Mg^{++} to tetracyclines of 25,000:1 or greater. At 1×10^{-3} M Mg^{++} and 2×10^{-5} M tetracycline 90 per cent of the antibiotic might be in complexed form. Since Ni^{++}, Co^{++}, and Zn^{++} form complexes of even lower dissociability but do not antagonize the action of tetracyclines (243b), simple removal of the drug by complex formation is not the whole story.

Uncoupling by tetracyclines, which has been studied almost entirely with enzymes from mammalian tissues, occurs only with concentrations (100–300 microgm./ml.) which are much higher than those which prevent multiplication of bacteria. Therefore, this action would appear to be involved in the antibacterial effects of these drugs only if the drug were concentrated by the bacteria, or

if the enzymes of aerobic phosphorylation in bacteria were much more sensitive to the antibiotic, or if bacteria were much more permeable to the drug than are mitochondria. Although Miura *et al.* (175) have reported that tetracyclines (at 1 microgm./ml.) specifically reduce P^{32} incorporation into the acid-soluble phosphorus fraction in bacterial cells, the experiments of Gale's group (reviewed, 67) indicate that the ability to concentrate glutamate (*Micrococcus pyogenes* var. *aureus*), an energy-requiring process, is not inhibited until about 100 microgm./ml. of drug is reached. This suggests that concentrations causing uncoupling in bacteria are as high as those effective with mitochondria.

With *Micrococcus pyogenes* var. *aureus* Gale and coworkers (67) have shown that concentrations which inhibit growth (1 microgm./ml.) also inhibit protein synthesis, while nucleic acid synthesis, certainly an energy-requiring process, may be doubled. Therefore, it seems unlikely that aerobic phosphorylation is the mechanism affected by low concentrations of tetracyclines. However, when high concentrations contact either bacterial or mammalian cells, as in the intestine, uncoupling may be of considerable significance. Large amounts of tetracycline do cause fatty livers and have been shown to interfere with fatty acid oxidation in liver preparations, probably by uncoupling (34).

X. CONCLUSION

It is hoped the foregoing may illustrate not only the complexities of drug-enzyme interactions but also the richness of the possibilities for selective interference with enzyme systems by foreign agents. At the present time, most of the successful drugs are developed empirically or through synthesis of congeners of pre-existing successful types. One wishes for a rational approach which would permit the deliberate development of new drugs specifically to affect particular enzymes. There have been many attempts to develop drugs on a rational basis. Perhaps these attempts will be more successful in the near future. But it must be admitted that the total enzyme matrix of the living organism is more complex and subtle than formerly visualized. Although new information concerning enzymes and their sensitivity to inhibition is being obtained at a constantly accelerated rate, each increment of knowledge also shows the goal to be a little further away than it had appeared to be.

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